OUTLINES OF BIOCHEMISTRY

THE ORGANIC CHEMISTRY AND THE PHYSICOCHEMICAL REACTIONS OF BIOLOGICALLY IMPORTANT COMPOUNDS AND SYSTEMS

BY

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THIS VOLUME IS DEDICATED TO

MY COLLEAGUES

TO THOSE PERSONS, PAST AND PRESENT, WHO HAVE BEEN ASSOCIATED WITH ME ON THE STAFF OF THE DIVISION OF AGRICULTURAL BIOCHEMISTRY IN THE UNIVERSITY OF MINNESOTA; FOR TO EACH AND EVERY ONE I OWE MUCH, AND IT IS ONLY THE SPLENDID COOPERATIVE SPIRIT OF THIS GROUP THAT HAS MADE THIS VOLUME POSSIBLE.

PREFACE TO THE SECOND EDITION

Another world is ours, we built a stately edifice
Set hard upon the solid ground of Nature.
Not a building made with hands.
Nor is it finished, mayhap it never will be finished;
Mayhap it takes a hundred centuries
To finish it, as in the past the craftsmen
Took a hundred years to build a great cathedral.
For we ask,
What is this MATTER that we see
And touch and weigh and measure?
What are these FORCES which we use and
Nature uses in her darkly hidden plans?
Whence came this universe of stars and space
And nebulae and atoms that we face?
How large is it?
How did it start? And when?
And whither does it tend?
Is it eternal? Or
Must it come at last to nothingness?
And what is life? Is it inevitable,
Given chemic elements and radiant energy
In due proportion?

Such questions do we set ourselves to answer,
Nothing less. And from the hard-sought answers
Build we up a structure wide as earth.
'Tis not a building made with hands.
It hardly seems a building. Yet it has
Some features such as buildings have.
For, here are stones in deep foundations laid
And only pebbles show above the ground.
There, from other deeps as firmly set
Rise giant towers flinging graceful buttresses
Afar in raceful arcs. And up
Into the blue and purple sky of thought
Extend the stately columns of accomplishment
In searching out the structure of the Universe.
Here a stone of metric properties is put in place.
There a lowly builder turns and turns a little stone
To fit into the scheme of things—and fails—
When slowly to his side come other builders
And behold—the little stone by
T'alchemy of fact and thought
Becomes a larger stone that fits. It fits—
It fits into the structure we are building.

Excerpt from "Appreciation" by OLIVER JUSTIN LEE, *Director of Dearborn Observatory, Northwestern University.*

In the nine years since the appearance of "Outlines of Biochemistry" there has been remarkable progress in the physicochemical interpretation of the reactions of biochemical systems and in the elucidation of the organic-chemical structure of many important cellular constituents. Then the vitamins were mysterious bio-catalysts; today not only are the structures of a number of the vitamins known with certainty but some have been synthesized in the chemical laboratory and are available commercially. Then we regarded the sterols as an isolated group of cellular constituents; today we know their structure and are amazed to find that the essential nucleus of the sterols is also the essential nucleus of the bile acids, the saponins, the cardiac glycosides, the toad poisons, the sex hormones, the cortical hormone of the adrenals, the vitamins D, and the carcinogenetic hydrocarbons. The presence of a hydrogen "here," or an oxygen "there," or a shift in the double bond, or a slight modification of an aliphatic side chain may change a compound essential to life-processes into a poison more potent than strychnine.

Similar advances have taken place in almost every field of interest to the biochemist, so that the present edition represents an extensive revision and in a large part the complete rewriting of the text. In addition, three new chapters have been added dealing, respectively, with oxidation-reduction, the flavins, and the hormones. A section on lignin has also been added.

The author wishes to emphasize again that he has attempted to give proper credit to other investigators for ideas or data which have been utilized, but that, since this volume does not pretend to be an exhaustive monographic treatment of the various topics, the literature cited may not be in historical sequence, nor may it represent the first publications on the specific topic. Each citation contains *something* that the author wishes to call to the attention of the prospective student, for this book was prepared primarily to assist graduate students, interested in biological problems, in orienting themselves in the field of biochemistry, and particularly to point out what potent tools organic chemistry and physical chemistry provide for the study of vital phenomena.

In conclusion, the author wishes to thank his colleagues, Dr. H. B. Bull, Dr. J. J. Willaman, and Dr. L. S. Palmer, for preparing the chapters on oxidation-reduction, the pectic substances, and the vitamins, respectively; other colleagues, Dr. C. H. Bailey, Dr. D. R. Briggs, and Dr. W. M. Sandstrom, for reading and criticizing certain chapters, also Dr. H. S. Isbell, of the United States Bureau of Standards, for assistance in certain interpretations of carbohydrate behavior; the Editors of the Journal of Biological Chemistry, the Journal of Physical Chemistry and The Williams and Wilkins Company, The Journal of Experimental Medicine, and Dr. Karl Landsteiner

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January, 1938

PREFACE TO THE FIRST EDITION

"When I have the honor of being consulted by a young man who has not yet found himself intellectually but who is filled with the desire to devote his life to some branch of medicine, be it clinical medicine, pathology, hygiene, bacteriology, physiology or pharmacology, my advice always is, 'Study chemistry at least three years. Try with all your power to master enough of this great science to start you on your career.'"

JOHN J. ABEL.

It is rather generally agreed among the scientists that the actions and reactions of a biological organism are expressions of the energy relationships due to chemical and physicochemical processes taking place within the cells and tissues which comprise the organism.

The biological organism can be looked upon as a complex system of chemical constituents, composed mainly of proteins, carbohydrates, fats and lipids, mineral elements, and water, which are organized by the mysterious forces which we call "life," and the actions and reactions of this protoplasmic mass are in turn determined by the energy interchanges of molecular transformations and surface and interfacial forces.

In most of the universities of America the development of the field of biochemistry has been left very largely to the group interested in the medical aspects. Accordingly, in a very large measure the biochemistry of the American universities is not biochemistry in its strictest sense, but rather leans more and more toward the field of human pathology. Most medical school biochemistry could be more correctly designated as human pathological chemistry.

It is obvious that there should be strong departments of physiological chemistry associated with the medical schools. However, it should likewise be recognized that there is a necessity for a study of the fundamental reactions underlying the broader field of biology, the primary object of which is to study and investigate the chemical and physicochemical reactions which take place in the normal biological organism, whether that organism be animal or plant.

The greatest advance in the biological sciences can take place only when the chemists are fully aware of certain of the biological problems and the biological point of view, and only when the biologists appreciate the assistance which chemical knowledge and chemical technic can offer to the solution of the major problems.

When, in 1913, Professor R. W. Thatcher was called to the University of Minnesota, he inaugurated a course of lectures on "phytochemistry" and taught the course until he became Dean of the Department of Agriculture in 1917, at which time Dr. C. A. Morrow assumed charge of the lecture work in "Phytochemistry" and supervision of the parallel laboratory course in "Biochemical Laboratory Methods."

Dr. Morrow remained in charge of both courses until his unfortunate illness in the spring of 1922, following which illness the responsibility for the lecture work was assumed by the present author, and upon resumption of active duties Dr. Morrow was left free to devote his entire energies to the development of the associated laboratory phases of the work. His success in this task is attested by the practical completion of the manuscript of the laboratory manual, "Biochemical Laboratory Methods," which appeared posthumously from the press of John Wiley & Sons, Inc., in 1927.

The course of lectures upon which the present volume is based must not therefore be regarded as the product of a single individual, for it embodies primarily the efforts of three men who have actually taught the course, and in addition the advice, cooperation, and criticism of all other persons who from time to time have been members of the Staff of the Division of Agricultural Biochemistry.

During the year 1925–1926 the present author prepared a series of mimeographed lecture outlines as an aid to the students in the classroom. These outlines were somewhat revised in the summer of 1927 and issued to the students in bound mimeographed form. The present volume follows, in general, the topics as presented in these outlines, although the scope of the field has been greatly expanded.

All of the reactions and interactions which we call life take place in a colloid system, and the author believes that much of the "vital energy" can in the last analysis be traced back to energies characteristic of surface films and interfaces. This belief is the justification for the detailed consideration of colloid systems which forms the first section of the volume.

In the subsequent sections dealing with proteins, carbohydrates, fats, etc., those organic compounds characteristic of living tissues, particular attention has been paid to structural organic chemistry and organic and physicochemical reactions. No attempt has been made to produce a "handbook" of biochemical compounds or a "descriptive biochemistry" detailing the chemical properties, crystal structure, etc., of the various compounds. There are already many volumes covering these fields, and no good purpose would be served by adding to the list.

The purpose of the present volume is that those students who are interested in biological phenomena may have an insight into the roles

which organic chemistry and physical chemistry play in living processes. It may be regarded as an attempt to interpret some of the reactions characteristic of the normal cell. Although a great many of the illustrations have been drawn from plant material, it must be remembered that in the last analysis the chemistry of the cell is essentially the same both for plants and for animals. There is no sharp distinction between "phytochemistry" and "zoochemistry." The same general reactions of protoplasm apply to both. If the student interested in some pure or applied field of biology or of chemistry is assisted, ever so little, in the clarification of the problems in his special field, then the object of the author will have been attained.

Toward this end the exact titles have been included in the literature citations. With but few exceptions (and those are noted in the text) the citations have been checked back against the original publication so as to preclude bibliographic errors. A sincere attempt has been made to give to other investigators the proper credit for data or for ideas which have been utilized. On the other hand, the text does not pretend to be an exhaustive treatment of the subjects, nor is the literature cited necessarily in an historical sequence. It is recognized that in many instances the references cited are not the first publications on the subject, but it is felt that the references which are cited contain something which should be called to the attention of the reader. The author may perhaps be pardoned for the numerous references to work done in his own laboratories, for obviously he is most familiar with the details of such work and the conditions under which it was carried out.

In conclusion, the author wishes to thank his colleagues, Dr. J. J. Willaman and Dr. Leroy S. Palmer, who have contributed Chapters XXVII and XXXIV, respectively; The Williams & Wilkins Company, and Prof. W. Mansfield Clark; the editors of the Journal of Biological Chemistry; The Chemical Catalog Company, Prof. Harry N. Holmes, Prof. O. L. Sponsler, and Prof. W. H. Dore; The Carnegie Institution of Washington, and Dr. D. T. MacDougal; Carl Zeiss, Inc.; Dr. Karl Mez; Prof. John J. Abel; Prof. E. F. Burton; Jerome Alexander; Prof. Francis Lloyd; Dr. Robert Newton; Dr. G. E. Holm; Dr. P. V. Wells; Dr. William Robinson, and others for permission to use copyrighted material or for photographs or data.

Parts of the manuscript have been carefully read and criticized by my colleagues, Dr. W. M. Sandstrom, Dr. J. J. Willaman, Dr. L. S. Palmer, Dr. C. H. Bailey, Dr. W. B. Sinclair, Dr. David R. Briggs, and Mr. Charles F. Rogers. To them I express my thanks.

And lastly I wish to express my deepest appreciation to Miss Rachel Rude for invaluable assistance in the task of preparing the final manuscript and in proofreading.

Ross Aiken Gortner

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ABBREVIATIONS AND SYMBOLS

The abbreviations used in the literature citations are, in general, those used in *Chemical Abstracts*. The symbols used in the mathematical formulas follow, so far as possible, those recommended in *International Critical Tables*, Vol. I, pp. 16–17.

I

COLLOIDS

"The colloidal is, in fact, the dynamical state of matter; crystalloidal being the statical condition. The colloid possesses ENERGIA. *It may be looked upon as the probable primary source of the force appearing in the phenomena of vitality."*

THOMAS GRAHAM (1861)

"The colloid field today presents the most promising realm in medical research."

W. G. MAYO (1928)

"If the colloid structures did not display highly specialized molecular structures at their surface, no reactions would occur; for here catalysis occurs. Were it not equipped with catalysts, every living unit would be a static system."

SIR FREDERICK GOWLAND HOPKINS (1933)

OUTLINES OF BIOCHEMISTRY

CHAPTER I

THE COLLOID STATE OF MATTER

It seems appropriate to begin the study of biochemistry with a consideration of the colloid state of matter, because in the last analysis many of the reactions of biological systems are dependent upon the colloidal phenomena operating in such systems. It is recommended that the beginner read "Theoretical and Applied Colloid Chemistry" by Wo. Ostwald, as translated by Martin Fischer.[1] This volume represents five lectures delivered by Professor Ostwald during a tour of the United States and Canada in the winter of 1913 and 1914. These lectures were prepared for the average reader, rather than for the specialist, and accordingly furnish a bird's-eye view of certain of the general phenomena characteristic of the colloid state. Incidentally the reader will become acquainted with some of the special nomenclature in this field.

We often hear the statement that such and such a material is a colloid. Such a statement is not strictly correct. Colloid phenomena deal with a *state* of matter, not a kind of matter. Early workers in this field published many papers indicating that certain materials could under certain specific conditions of manipulation be brought into the colloidal state. We now know that, using the proper technic, any material can be brought into the colloidal state. Consequently there is no sharp line of demarcation between substances which can be regarded as colloids and substances which are generally non-colloids.

The first real work in the field of colloid chemistry was due to the activities of Thomas Graham (1805–1869). All of Graham's forty-six research papers dealt with the single phenomenon of diffusion.[2] Graham [3, 4] published important summaries in 1861 and 1864, and in these introduced many new terms. Thus, speaking of the properties of col-

[1] John Wiley & Sons, New York (1917).

[2] Cf. Gortner, R. A., Colloids in Biochemistry. An Appreciation of Thomas Graham, *J. Chem. Educ.*, 11: 279–283 (1934).

[3] Graham, Thomas, Liquid Diffusion Applied to Analysis, *Phil. Trans.*, 1861, pp. 183–224.

[4] Graham, Thomas, On the Properties of Silicic Acid and Other Analogous Colloidal Substances, *J. Chem. Soc.*, 17: 318–327 (1864).

loids, Graham states, "Their peculiar physical aggregation with the chemical indifference referred to appears to be required in substances that can intervene in the organic processes of life. The plastic elements of the animal body are found in this class. As gelatine appears to be its type, it is proposed to designate substances of the class as *colloidal*[5] and to speak of their peculiar form of aggregation as the *colloidal condition of matter*. Opposed to the colloidal is the crystalline condition. Substances affecting the latter form will be classed as *crystalloids*. The discussion is no doubt one of intimate molecular constitution."

Graham recognized that there was no sharp discontinuity between colloids and crystalloids and that one and the same substance may under different sets of conditions be either colloidal or crystalloidal. Thus, he states (1861), "A departure from its normal condition appears to be presented by a colloid holding so high a place in its class as albumen. In the so-called blood-crystals of Funke, a soft and gelatinous albuminoid body is seen to assume a crystalline contour. Can any facts more strikingly illustrate the maxim that in nature there are no abrupt transitions, and that distinctions of class are never absolute?"

We now know that any substance, even including such substances as sodium chloride, can by proper technic and by the proper choice of medium be brought into the colloidal state. Some substances may be colloidal in one liquid medium and truly crystalloidal in another. Tannin dispersed in water gives rise to a colloidal system, whereas it dissolves in acetic acid to form a crystalloidal solution. Many other substances show similar behavior. *Only the physical properties of the resulting system will show whether or not a given material is colloidal or crystalloidal.*

A substance cannot be strictly spoken of as a colloid, for the colloid implies (1) a state of matter, and (2) at least two components or phases. Thus, we have not a colloid material but rather colloidal systems.

Water has three states, vapor, liquid, and solid. Each of these may exist in colloidal systems. Barnes[6] has discussed certain natural phenomena produced by colloidal water and colloidal ice, and we shall have occasion later to consider instances where water vapor is involved.

Inasmuch as a colloid system is a heterogeneous system, it is necessary to distinguish between the substance which is dispersed and the medium in which the material is dispersed. Various terms have been employed by the different writers. Some authors speak of the *disperse*

[5] From the Greek, κολλα, kolla, meaning glue or gelatin, and ειδοσ, eidos, meaning like.

[6] Barnes, Howard T., Colloidal Water and Ice, Colloid Symposium Monograph, Vol. III, pp. 103–111, Chemical Catalog Company, New York (1925).

phase and *dispersions medium*; others of the *discontinuous phase* and the *continuous phase*; others of the *internal phase* and the *external phase*; and still others of the *micelles* and the *intermicellar liquid.* The last group of terms appears to be coming more and more into general use and in some respects appears preferable.

Martin Fischer has given a definition of a colloidal system which is fairly satisfactory. He states that "colloid systems result whenever one material is divided into a second with a degree of subdivision coarser than molecular." This definition, though approximate, is not strictly true. The definition could probably be restated with minor changes so as to hold rigidly, *i.e., colloidal systems result whenever one material is divided into a second with a degree of subdivision either (a) coarser than molecular or (b) where the micelles exceed* 1–1.5 $m\mu$ *in diameter.* A graphic representation of the field of colloid chemistry is shown in the following diagram:

Molecules and Ions	Colloids	Matter in Mass
Not Visible in Ultramicroscope	Visible in Ultramicroscope	Visible in Microscope
1 $m\mu$	0.1 μ	

It will be noted that an arbitrary boundary has been placed at 1 $m\mu$ (0.000001 mm.) defining the lower limit of the colloid realm, and another arbitrary boundary at approximately 0.1 μ (0.0001 mm.) as defining the upper limit of the colloid realm. It cannot be too strongly emphasized that *these are arbitrary boundaries.* However, they seem to have been rather wisely chosen. Most compounds which are crystalline in the solid state and which yield mono-disperse solutions of molecules or ions rarely contain molecules which exceed 1 $m\mu$ in diameter. In some instances, for example, certain of the crystalline proteins, and specifically in egg albumin, we have truly monomolecular solutions which at the same time exhibit certain properties of colloidal systems. This phenomenon is not in disagreement with the second part of our definition of a colloid system. We are probably dealing in such cases with massive molecules, perhaps 5 to 10 $m\mu$ in diameter.

The upper limit of the colloid realm, 0.1 μ in diameter, was chosen because this represents the extreme range of the ordinary microscope, using an oil-immersion objective. The colloid realm is thus concerned with systems containing subdivisions of matter lying between the limits of visibility of the usual laboratory microscope and those solutions which are strictly crystalloidal. It must be understood at the outset that the properties of the colloidal systems do not strictly coincide with these arbitrary boundaries but that *there is a continual gradation in properties from truly crystalloidal systems to coarsely divided suspensions, and that the optimum zone of colloidality lies somewhere near*

the center of the colloid realm, as noted in the diagram above. In other words a coarsely divided suspension will show to a minor degree certain of the phenomena characteristic of the colloidal state, and in the same way certain of the phenomena of the colloidal state reflect in a minor degree the characteristics of true solutions.

Siedentopf and Zsigmondy classify the three states of matter noted in the above diagram as *microns* visible in the microscope, *submicrons* or *ultramicrons* visible in the ultramicroscope, and *amicrons* not visible in the ultramicroscope. According to their terminology all systems showing characteristic colloidal properties contain submicrons. We are probably nearer the truth when we place the lower limit of colloid systems at 1 mμ, than when we place the upper limit at 0.1 μ, and there has been a general tendency of recent years to raise the upper limit to perhaps 0.5 μ, inasmuch as fine suspensions possess to a large degree certain characteristic properties of colloidal systems.

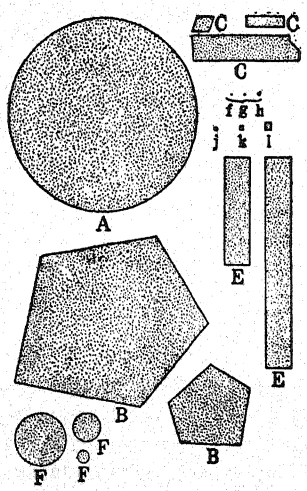


FIG. 1.—A = red blood cells; B = starch grains; C = clay particles; E = *Anthrax bacilli; F* = *Cocci; fgh* = colloidal gold (f = 10 mμ); *jkl* = gold coagula. (After Zsigmondy.) By permission from Alexander's (1924) "Colloid Chemistry."

Perhaps everyone has viewed a red blood corpuscle under the microscope and accordingly has some idea of the relative size of a red blood corpuscle as compared with other familiar objects. Figure 1 illustrates the size relationships of some rather common materials as compared with the size of colloidal particles. It should be noted, however, that the smallest colloidal particle noted at (f) is ten times the diameter of the particles which fix the lower limit of the colloidal realm. Hackh [7] has tabulated some very interesting data comparing size, time, mass, velocity, and temperature relationships for common objects and systems. A study of these tabulations will greatly assist the student in orienting himself in the field of natural phenomena.

According to Wo. Ostwald one can conveniently classify colloid systems into eight great groups:

1. SOLID-IN-SOLID.—Examples are the ruby glass of the cathedral windows which is a colloid system of metallic gold dispersed in glass; blue rock salt, a colloid system of sodium dispersed in sodium chloride; the black diamond, which is carbon dispersed in crystalline carbon. The colors of many precious stones are examples of solid-in-solid col-

[7] Hackh, I. W. D., Some Magnitudes, *Scientific Monthly,* 36: 55–61 (1933).

loidal systems. This type of system is important in metallurgy, but from the biological standpoint is relatively unimportant.

2. SOLID-IN-LIQUID.—Faraday, in 1857, exhibited before the Royal Society a brilliant red liquid which he had prepared by reducing an aqueous solution of auric chloride. Inasmuch as this liquid showed a beautiful red color by transmitted light and a golden sheen by reflected light, he suggested that the color might be due to particles of solid gold suspended in the liquid. This experiment was forgotten for many years, but we now know that Faraday had prepared one of the first examples of a solid-in-liquid colloidal system.

Wo. Ostwald has given to this class of colloid systems the specific name of *suspensoids*. However, the definition of "a solid-in-liquid" does not at all times tell the true story. In the gold-in-water systems we have a true solid-in-liquid, but it is easy to prepare a system having similar properties by the dispersion of an oil in water. Both preparations would according to Ostwald's terminology be called suspensoid systems. Perhaps a better terminology would be a *lyophobic* system, and a better definition for systems showing lyophobic behavior would be that suggested by Martin Fischer, namely, *a system in which the disperse phase is insoluble in the dispersions medium and the dispersions medium does not dissolve in the disperse phase*. Thus, we may have systems of gold and water, sulfur and water, rubber and water, sodium chloride and alcohol, etc. This type of system is of rather general importance in biochemical studies, and certain of its characteristics will be considered in greater detail in the following pages.

3. SOLID-IN-GAS.—In this system we are dealing with such phenomena as the smoke-vapors of ammonia and hydrochloric acid, the "blue haze" of the forest fire areas that hangs for weeks in the air without settling, and the "blue" smoke of the cigarette. The darker colored smokes usually contain the more coarsely divided suspensions of carbon. When these larger particles have settled out, the blue colloidal haze still persists and forms a very stable system. From the military standpoint such systems constitute a rather important field of study involving smoke screens, etc., for the reason that one desires the maximum density of smoke which can be formed from the minimum amount of original material, the degree of subdivision thus playing a very important role. Bacterial and fungus spores present in spray from coughing and sneezing may be considered as examples of solid-in-gas systems which are of importance from the biological and medical standpoint.

4. LIQUID-IN-SOLID.—The principal examples of this system are to be found in minerals and gems. The opal is a system of silicon dioxide and water, the pearl a system of calcium carbonate and water. An opal rather readily loses its "fire" and the pearl its "life" or lustre if kept for a considerable period of time in a dry atmosphere. In-

stances have been known where valuable pearls placed for years in a safe-deposit box have been rendered practically valueless. Both the opal and the pearl are most beautiful when worn often near the skin, *i.e.*, in a region of fairly high humidity tending to keep the equilibrium amount of water in the gem.

5. LIQUID-IN-LIQUID.—This class Wo. Ostwald calls the *emulsoids*, but it is generally referred to as the *lyophilic colloids*. Here again the "liquid-in-liquid" terminology is not strictly accurate. A far better definition is that of Martin Fischer who defines this group as "*a system in which the disperse phase and the dispersions medium are mutually more or less soluble one in the other.*" This indicates that *hydration* (when water is the dispersions medium) or *solvation* (a term which applies to any dispersions medium) takes place. Typical examples are gelatin and water. Gelatin swells in water; some of the gelatin disperses in the water, and a considerable amount of the water "dissolves" in the gelatin. The gelatin may be a liquid particle or it may be a solid particle. We do not know which condition actually exists, but we do know that gelatin in water is *hydrated*, and that is the important point. Gelatin in water is a lyophilic (solvent-loving) system. Gelatin in alcohol or benzene is a lyophobic system. Rubber in alcohol or benzene is a lyophilic system, in water a lyophobic system. In biological problems this class of lyophilic colloids is by far the most important class.

6. LIQUID-IN-GAS.—FOGS, MISTS.—This class is important from the standpoint of meteorology. In fogs and mists we probably deal with a solid-in-gas, with water particles condensed on the solid surfaces. Owens [8] studied conditions which bring about fog in the London area. He finds that on a clear day there is approximately 1 mg. of solid material per cubic meter of air. In a dense fog the amount of solid material rises to the neighborhood of 5 mg. of solid material per cubic meter of air. This does not seem a large amount, but over the London area it amounts to 193 tons of solid material on the 120 square miles of area to a height of 122 meters. The size of the solid particles varies from 0.00013 to 0.00026 mm. in diameter. The water film condensed on the surface of these solid particles may be as great as 0.0014 mm. in thickness. Owens notes that at 6 A.M. the air above London may be perfectly clear and at 9 A.M. there may be a dense fog, and he ascribes the onset of the fog to the smoke rising from the fires of the homes and factories. In the Ninth Report on Atmospheric Pollution [9] it is

[8] Owens, Seventh Report of the Committee for the Investigation of Atmospheric Pollution, No. 249, Meteorological Office, Air Ministry, London, 1922. (See Science, 55: 596–597, 1922.)

[9] Ninth Report of the Committee for the Investigation of Atmospheric Pollution. Report on Observations in the year ended March 31, 1923, Meteorological Office, London (1924). Abstracted in *Science*, 60: 136 (1924).

pointed out that on a clear day there are approximately 100 particles of solid per cubic centimeter of air, whereas in a dense fog there may be as many as 80,000 particles. Incidentally it may be noted that 10,000 particles of solid per cubic centimeter are equivalent to a weight of 1 mg. per cubic meter. The diameters of the particles range from 1.5 μ down to ultramicroscope size. In a dense London fog a count of 53,000 particles per cubic centimeter of air was made. The solid content of air is a rather important biological problem. Under London conditions a person would inhale 500 billion particles in a period of twenty-four hours, or enough particles to make a string 250 miles long. A considerable part of these is removed by the cilia and cells of the lungs, but a very substantial part is retained. Pathologists are often able from post-mortem examinations to differentiate between urban and rural dwellers. Adults from rural districts usually have pink lungs, whereas city dwellers almost invariably have lungs which are dark brown and in many instances as black as charcoal. In the pathological museum of the University of Chicago there are exhibits of city dwellers' lungs and of material removed from such lungs. In one instance there is a test-tube almost completely full of black carbon which was removed from one lung of a city dweller. The corresponding opposite lung is on exhibit and is dense black.

London is cited as a typical example of a modern city. The amount of solid material deposited from the air per month in certain of our own cities has been recorded [10] as follows:

		Tons
Pittsburgh,	April, 1923	115.6
	April, 1912	98.7
	April, 1916	109.8
St. Louis,	April, 1916	59.6
Cincinnati,	April, 1916	52.0

Many important aspects of the dust, smoke, and fog problems were recently considered in a general discussion [11] of the Faraday Society. Our own recent dust storms in the western area emphasize the importance of such systems to the problems of geography, agriculture, and medicine. In the latter field mention may be made of numerous cases of silicosis which have developed as the result of the inhalation of silica.

7. GAS-IN-SOLIDS.—From the biological standpoint this field is relatively unimportant. From the standpoint of mineralogy, metallurgy, and industrial chemistry it offers a fruitful field of research. The form

[10] Meller, H. B., Economy through Smoke Abatement, *Ind. Eng. Chem.*, **16**: 1049–1051 (1924).

[11] Disperse Systems in Gases: Dust, Smoke, and Fog (Sixty-fourth General Discussion of the Faraday Society), *Trans. Faraday Soc.*, 32: 1041–1300 (1936).

in which gas is present in solids is still in many instances a debatable question. In some minerals gas probably occurs in the form of very finely divided bubbles, the bubbles perhaps being of colloidal dimensions. In other instances the gas is probably adsorbed or "fixed" on the surface of the solid material. Many solid materials possess the property of condensing or adsorbing gas upon their surfaces, and a great variety of industrial applications have been based upon this phenomenon. Possibly such systems could be logically spoken of as gas-in-solid systems. However, this is not the place for an extended discussion of such systems, which will be reserved for consideration later under adsorption.

8. GAS-IN-LIQUID.—Such systems are generally spoken of as foams or systems in which gas bubbles are finely divided and suspended in liquids. The liquid in which the gas bubbles are suspended is usually not a pure material but is more often already a colloidal system containing lyophilic colloids. Typical foams are produced when egg white is beaten or when cream is whipped, the lyophilic colloids which are present serving to stabilize the resulting foam. In many instances foams which are extremely difficult to break develop in industrial processes. Accordingly a study of such systems is necessary in order to devise methods for the destruction of foams at points where they are undesirable.

9. GAS-IN-GAS.—The foregoing eight classes of colloidal systems have all been realized experimentally and all occur in nature. The ninth hypothetical system, gas-in-gas, is theoretically impossible to attain, inasmuch as gas is always regarded as occurring in the molecular state, so that large aggregates of molecules are theoretically impossible, for no known gas molecule is of colloidal dimensions. Possibly if someone were to work with mixtures of gases at their critical state, this system might be experimentally realized. It is doubtful, however, whether it would be of any technical importance. Certainly it would have no biological significance.

EMULSIONS.—Emulsions are not necessarily truly colloidal systems, if we limit our definition to the size of the particle concerned. Emulsions may be defined as systems of oil dispersed in water or as systems of water dispersed in oil, although in many instances the dispersions medium contains a lyophilic colloid to assist in stabilizing the emulsion. Very dilute emulsions could be classified as liquid-in-liquid systems, providing all the particles fell within the range of diameters characteristic of colloidal systems. Most emulsions, however, contain oil or water droplets very much larger than even the 0.5 μ, which forms the upper limit of the truly colloidal field. Mayonnaise, milk, and egg yolk are typical examples of emulsions. In each of the examples we are dealing not only with oil droplets suspended in an aqueous medium, but we have present at the same time lyophilic colloids in the form of

proteins which stabilize the emulsion, so that a study of emulsions involves not only a study of the dispersions medium and the disperse phase, but likewise, and probably of greater importance, an investigation of the nature and properties of the lyophilic colloid which acts as the stabilizer.

Protoplasm has been spoken of as an emulsion. Certainly there are droplets of fats and oils in living protoplasm which are stabilized by the lyophilic colloids which are present. It is equally true that there are solid particles in living protoplasm, so that protoplasm can be looked upon as a complex colloid system, the dispersions medium being water and the disperse phases consisting of lyophilic colloids, lyophobic colloids, and microscopically visible fat droplets in the form of an emulsion as well as other microscopically visible particles.

NOMENCLATURE.—It is necessary at this point to consider a few general terms of special nomenclature.

Graham designated liquid colloidal systems as *sols*. A sol may be defined as a colloidal system which to the eye appears as a solution, *i.e.*, it is fluid and appears to be homogeneous. It can be more or less readily poured from one beaker to another, but it differs from a solution in that the sizes of particles suspended in the liquid are of colloidal dimensions. Occasionally in speaking of sols, it is desirable to define the dispersions medium. Thus, following Graham's terminology, we have hydrosols where water is the dispersions medium, alcosols, benzosols, etc., with organosols as a special term limited to organic solvents as a dispersions medium.

Graham designated the more or less rigid colloidal systems as *gels*. A gel may be defined as a colloidal system possessing more or less the properties of a solid. Gels logically fall into two general subdivisions.

1. The true gels or "jellies," as some prefer to call them, are formed by the characteristically lyophilic colloids and may be represented by such familiar materials as gelatin, thick custards, the ordinary fruit jellies and jams of the household, muscle tissue, heat-coagulated egg white, etc., and from the biological standpoint constitute the important group of gels.

2. Another subdivision, sometimes referred to as gels, is the precipitates which are formed by the coagulation of lyophobic colloid systems. These are preferably referred to as *coagula* rather than as gels, although there is no general agreement in regard to this distinction in nomenclature. When a gold hydrosol is precipitated by the addition of an electrolyte (*vide infra*), a purplish precipitate is thrown down. This precipitate is relatively bulky, and when this precipitate is ignited, it is often found that an almost unweighable amount of gold is actually present. The precipitate contains the gold particles originally dispersed in the form of a sol, and adhering to these coagulated particles is a relatively large amount of the dispersions medium

(water). Inasmuch as such a precipitate does not show, in general, properties similar to those exhibited by the "jellies," it seems preferable to differentiate between the two types and to refer to the precipitates formed in lyophobic systems as *coagula.*

In the gels as in the sols, we use such special terminology as hydrogels, alcogels, benzogels, organogels, etc.

The terms lyophilic and lyophobic have already been defined; they are general terms referring to the affinity or lack of affinity of the disperse phase for the dispersions medium. A lyophilic system in general produces a gel which contains a high percentage of the dispersions medium. A lyophobic system on the other hand yields a coagulum which contains a relatively small amount of the dispersions medium. A few exceptions to the above rules (for example, dibenzoyl-*l*-cystine gel) will be taken up later.

CHAPTER II

METHODS OF PREPARATION

The methods of preparation of colloidal systems are referred to in the literature under two separate systems of nomenclature. We have the classification due to Svedberg, which is used by Wo. Ostwald.

	Crystals or Matter in Mass	Colloid Systems	True Solution
Dispersion Methods →			Condensation Methods ←

Or we have the classification due to Von Weimarn.

	Crystals or Matter in Mass	Colloid Systems	True Solution
Solution Methods →			Crystallization Methods ←

These classifications are essentially identical, the significant feature of both being to illustrate the fact that there is a continuous gradation from matter in mass to true solutions. Every crystal which grows in a true solution has at one time possessed dimensions which would bring it within the colloid realm. Likewise every crystal which dissolves to form a true solution must eventually yield a particle of colloidal size before it completely disappears into true solution. Either Svedberg's or Von Weimarn's classification is satisfactory.

To prepare a stable colloidal sol one must stop when the particles are within the colloidal realm. It is also reasonably desirable to have fairly high concentration of the disperse phase in the dispersions medium.

When condensation methods are employed, the stability and the concentration of the resulting system depend upon two factors, (*a*) the velocity of the formation of nuclei, and (*b*) the rate of crystal growth. Von Weimarn has expressed these two phenomena in some rather general equations.

$$W = K \frac{\text{Condensation pressure}}{\text{Condensation resistance}} \qquad (1)$$

where W is the velocity of nuclei formation and K is a constant.

If C is the total amount in solution and L is the solubility $(C - L)$ is the condensation pressure or the supersaturation, and L, the solubility, is the condensation resistance. The equation then becomes

$$W = K\frac{(C - L)}{L} \tag{2}$$

This equation means that there is a certain tendency for the material to remain in a supersaturated solution expressed by condensation resistance and that the velocity is dependent upon the ratio of these two factors to each other and to a constant. It is obvious that, the more nuclei formed, the greater will be the surface area of the disperse phase, and likewise the smaller will be the diameter of the resulting particles per unit weight of material. To form a stable colloidal system it is, therefore, desirable to have as great a number of nuclei formed as is practicable.

Once the nuclei are formed, they grow according to the Noyes-Nernst equation for crystal growth:

$$V = \frac{\Delta}{l}S(C - L) \tag{3}$$

where V = velocity of growth;
Δ = diffusion coefficient;
l = length of diffusion path;
S = surface area of disperse phase;
C = concentration of solution;
L = solubility of disperse phase of a given size;
$(C - L)$ = absolute supersaturation.

As illustration let us consider silver chloride and sodium chloride; for sodium chloride $(C - L)$, the supersaturation, may be relatively great, and $([C - L]/L)$ or the excess of sodium chloride in a solution of the very soluble sodium chloride will make for a low velocity of nuclei formation and for the formation of relatively large crystals of sodium chloride. Accordingly a hydrosol of sodium chloride is not attainable. With the same values for $(C - L)$ for silver chloride we get at once a dense, curdy precipitate (a gel or coagulum) of silver chloride. For sodium chloride L was large, and slow crystallization resulted. For silver chloride L is extremely small, and instant precipitation occurs. If instead of using an aqueous medium we had formed the sodium chloride by the interaction of sodium ethylate dissolved in absolute alcohol and hydrogen chloride dissolved in absolute alcohol, we would have formed either a curdy precipitate, similar to the precipitate of silver chloride, or a stable colloidal system of a sodium chloride alcosol, depending upon the concentrations of the materials which were used. *The smaller the solubility of a substance in a solvent, the easier it is*

to prepare a colloidal system, and there is a maximum solubility above which a stable colloidal system, at least for lyophobic colloids, is impossible. Taylor[1] has discussed at greater length this phase of the preparation of sols, and the reader is referred to him for a more elaborate treatment; cf. also Bradford.[2]

Adding nuclei on which crystal growth will take place has been suggested.[3] In the instance cited a small drop of a sol of potassium zirconium sulfate was added to the solution under test in order to induce crystallization and turbidity sufficient to measure. By this modification the sensitivity of the zirconium sulfate reagent for potassium was increased from 0.48 mg. potassium to 0.32 mg. potassium per 2 cc. of reaction mixture.

The concentrations of the solutions determine in a large measure the nature of the system which will result. If one deals with concentrated solutions of very soluble substances which form a very insoluble precipitate, a colloidal gel is likely to result. Intermediate concentrations of the same materials may yield granular or crystalline precipitates which can be easily filtered off and which show no typical colloidal properties. Very dilute solutions of the same materials when mixed may yield an excellent colloidal sol. Figure 2 illustrates these generalizations in a diagrammatic way.

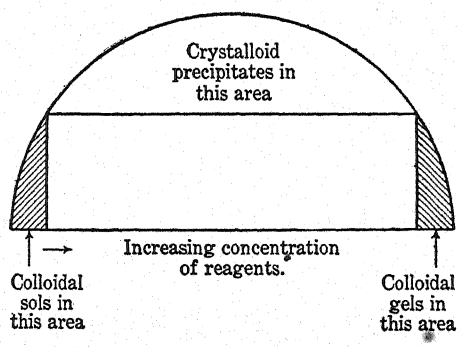


FIG. 2.—A diagrammatic representation of the role of reagent concentration on the properties of the resulting systems.

Typical examples are laboratory experiments, using ferric chloride and potassium ferrocyanide to form Prussian blue. If one employs equimolecular concentrated solutions and mixes these solutions practically instantaneously, the resulting Prussian blue forms a dense gel, and if a small portion of this gel is stirred into a large volume of pure water, a stable colloidal sol of Prussian blue results. Such a sol will readily pass unchanged through a filter paper. If now one dilutes

[1] Taylor, W. W., The Chemistry of Colloids, Second Edition, pp. 171–179, Longmans, Green and Company, New York (1915).

[2] Bradford, S. C., Dispersoidology and the Theory of von Weimarn. Some Remarks upon the Significance of the Crystal and Colloid States, *Sci. Am. Supplement*, No. 2255: 182–183 (March 22, 1919).

[3] Reed, R. D., and Withrow, J. R., Zirconium VI. Use of Dispersoids in the Detection of Traces of Potassium by Zirconium Sulfate, *J. Am. Chem. Soc.*, 52: 2666–2668 (1930).

these concentrated solutions eight or ten times and again mixes them, a precipitate of Prussian blue is formed which can be readily filtered off. If one further dilutes the initial solutions so as to have only a few milligrams of Prussian blue formed per 100 cc. of the resulting mixture, one obtains a stable sol of Prussian blue which again will readily pass through a filter paper. Accordingly if one wishes to filter off a precipitate of Prussian blue, the initial concentration of the reagents which are employed may be a determining factor in the complete retention of the precipitate on the filter. In the colorimetric estimation of hydrocyanic acid, it is essential that all the Prussian blue remain in the form of a colloidal sol. In such cases the initial concentration of the hydrocyanic acid solution must not exceed a certain value; the limit is probably near 4 mg. of hydrocyanic acid per 100 cc. Under such conditions the Prussian blue sol will be stable, and its depth of color can be accurately measured in a colorimeter. If much greater amounts of hydrocyanic acid are present, a granular or crystalline precipitate is likely to form, and such a precipitate will settle rather readily and give low results when the color is measured.

Similar experiments can be conducted by using ferric chloride and sodium arsenate or ferric chloride and sodium phosphate as the reacting materials. A series of beakers arranged with progressive dilutions of the reagents will show at one end of the series clear and apparently homogeneous gels and at the other end transparent, apparently homogeneous sols, while the intervening dilutions will show more or less complete precipitation grading in properties from one end of the series to the other. It is obvious that such phenomena are of extreme importance to the analytical chemist, and many of the instances where the precipitate "goes through the filter paper" can be avoided by choosing the proper concentration prior to making the precipitation.

A rather striking illustration of the influence of concentration of reagents is afforded by the data of Krienke.[4] The problem under consideration was to ascertain what effect the rate of addition of silver nitrate to an exactly equivalent solution of hydrochloric acid had on the occlusion of impurities in the resulting precipitate of silver chloride. The rate of addition of the silver nitrate was planned to be the only variable. As an indicator of the impurities carried down or adsorbed by the precipitate, use was made of a dilute solution of radium, inasmuch as extremely small quantities of radium can be determined with a very high degree of accuracy by the use of the electroscope. A known quantity of radium was added to the hydrochloric acid solution and then an exactly equivalent amount of silver nitrate added over various periods of time, the time of addition ranging from 1 sec-

[4] Krienke, O. K., The Difference in the Amount of Radioactive Impurity Carried Down by Rapid or Slow Precipitation, unpublished manuscript.

ond to 4,000 seconds. When these data were plotted in the form of curves, the coordinates being time of addition of the silver nitrate solution and the radioactivity of the resulting precipitate or of the supernatant liquor, curves similar to those in Fig. 3 resulted. The solid lines, *AB* and *DE*, represent the lines obtained from the experimental data, and the dotted line, *BD*, is inserted to indicate how the curve might have been drawn, providing that the experiment had a single variable, *i.e.*, the time of the addition of the silver nitrate. The experimental curve then would be represented by *ABDE*. It at first appeared as if experimental errors or some uncontrolled factor accounted for the abrupt change in form of the curve from *AB* to *D*. However, a repetition of the experiment invariably gave the same sort of data. A further study of the experimental conditions revealed the fact that Experiments 1 and 2 (the 1-second and 20-second times of additions) were performed by allowing the silver nitrate solution to flow from the tip of a 5-cc. pipet. It was impracticable to use a 5-cc. pipet to control the rate of flow for the longer periods, and accordingly a buret was employed, *the silver nitrate solution being diluted from a volume of 5 cc. to 55.5 cc.* with a correspondingly smaller amount of water being present in the hydrochloric acid solution to which the silver nitrate solution was added, although a final volume of 200 cc. was maintained in all experiments. The net effect of this change in experimental technic was to add a more dilute solution of silver nitrate to a more concentrated solution of hydrochloric acid, although there was no change in the actual weight of silver nitrate or hydrochloric acid which was used. The mere change in initial concentrations produced the appreciable change in the amount of radium carried down by the precipitate. Probably what actually happened was an alteration in the surface area of the resulting precipitated silver chloride, so that the precipitate formed on the addition of the more dilute silver nitrate had a greater surface area and consequently a greater adsorptive capacity. Referring to Fig. 2, the precipitating conditions were shifted from some point within the boundary of the crystalloidal field to a point nearer the boundary of the colloidal sols.

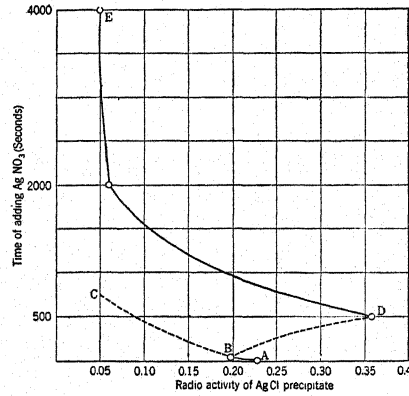


FIG. 3.—Showing the effect of the precipitating conditions on the occlusion of radioactive impurities in AgCl. (Data of Krienke.)

According to the above explanation, which admittedly involves assumptions which Krienke did not definitely prove, the dotted line *BD* in Fig. 3 is without justification, and instead the dotted line should have been an extension of the line *AB* to some point approximately in the neighborhood of *C*, and we would thus have two curves, *AC* and *DE*, more or less parallel to each other, representing the actual experimental conditions, *AC* representing an experiment in which 5 cc. of solution containing 0.8450 gram of silver nitrate was added, over varying periods of time, to 195 cc. of hydrochloric acid exactly equivalent to the silver nitrate which was added, and the curve *DE* representing the addition of 55.5 cc. of a solution containing the same amount of silver nitrate to 144.5 cc. of hydrochloric acid equivalent to the silver nitrate added. This series of experiments indicates the effect of concentration of reagents on the purity of the resulting precipitate and on the conditions which are necessary for complete precipitation and ease of filtration and washing of the precipitate.

Before we consider the various methods by which colloidal sols may be formed, it is necessary to note one additional property characteristic of stable colloidal systems. *The micelles present in colloidal systems possess either a positive or a negative electric charge.* The electric charge may arise from several causes such as the *direct ionization* of the material comprising the micelle, *the capture of an ion by the micelle* (adsorption, *vide infra*) in which case the micelle attracts to it an ion of an electrolyte and this, becoming fixed on the surface, gives rise to either a positive or a negative charge, or perhaps in rare instances *electrification by contact* with the dispersions medium, in the same way that a wax rod becomes charged when rubbed with a woolen cloth.

Most hydrosols contain negatively charged micelles, whereas sols where turpentine is the dispersions medium ordinarily contain positively charged micelles. The explanation for the reversal of charge when we pass from water to turpentine has been ascribed as being due to a change in the dielectric constant of the dispersions medium and has given rise to the general rule that *micelles are usually negative when in contact with a liquid having a high dielectric constant, and positive when in contact with a liquid having a low dielectric constant.* The dielectric constant of water is 81, which is very high as compared to most liquids. Hydrogen peroxide (46 per cent) is somewhat higher, 84.7. Certain of the dielectric constants of common liquids are shown in Table I.

As a general rule only the oxides and hydroxides of metals and basic organic compounds are positively charged in water.[5] In these instances we are probably dealing with either the phenomena of ioni-

[5] There are some exceptions to this, but as a generalization it is fairly satisfactory.

TABLE I

SHOWING THE DIELECTRIC CONSTANTS OF CERTAIN COMMON LIQUIDS

	Temperature, ° C.	Dielectric Constant		Temperature, ° C.	Dielectric Constant
Methyl alcohol	13.4	35.3	Carbon disulfide	17.0	2.64
Ethyl alcohol	14.7	26.8	Carbon tetrachloride	18.0	2.25
Glycerine	15.0	56.2	Benzene	19.0	2.26
Acetone	20.0	21.5	Turpentine	20.0	2.23
Acetic acid	18.0	9.7	Petroleum	21.0	2.12
Chloroform	18.0	5.2	Olive oil	21.0	3.11
Ethyl ether	18.0	4.368	Phenol	48.0	9.68

zation or of the adsorption of an ion. Thus, silver hydroxide or ferric hydroxide might be regarded as ionizing according to the scheme:

$$AgOH \rightarrow Ag^+ \text{ and } OH^-$$
$$[Fe(OH)_3]_x \rightarrow [Fe(OH)_2]_x^+ \text{ and } OH^-$$

Or we might postulate that the ferric hydroxide micelle still contained a small amount of ferric chloride, in which case we could have an ionization as follows:

$$[Fe(OH)_3]_x(FeCl_3)_y \rightarrow [Fe(OH)_3]_x(FeCl_2)_y^+ \text{ and } Cl^-$$

In each one of these instances we have a negative ion given off into the solution with a corresponding residual positive charge on the micelle. Other discussions of the electrical charge will be given in greater detail in the following pages. It may not be amiss, however, to discuss briefly the meaning of the term *dielectric constant.*

Hildebrand [6] states, "Suppose that we have two metal plates that can be charged electrically, one positive, the other negative. If this is done in a vacuum, there is a definite amount of electricity stored up on applying a given electric potential; however, if the space between the plates is filled with a given liquid, a larger amount of electricity is absorbed before the same potential is reached. *The ratio of the amount of electricity for this liquid to the amount in a vacuum is called its dielectric constant.*" Hildebrand then goes on to consider how this extra charge can be absorbed and indicates that there are three measurements which may be involved: (1) the electrons within the molecules may be displaced in their relative position to the positive atomic nuclei; (2) the molecules may be stretched, twisted, or bent by the

[6] Hildebrand, J. H., The Liquid State, *Science*, 80: 125–133 (1934).

displacement of the atoms within them; and (3) the molecules may become oriented providing they already possess permanent electron displacements or dipoles, and in this orientation the positive portion of the molecule may be brought closer to the negative plate, and *vice versa.*

Table II, from Hildebrand's paper, distributes the total polarization of four chemical compounds between these three measurements.

TABLE II

THE CONTRIBUTION OF ELECTRON DISPLACEMENT, ATOMIC DISPLACEMENT, AND MOLECULAR ORIENTATION TO THE MOLAL POLARIZATION OF CERTAIN CHEMICAL COMPOUNDS

Compound	Electron	Atomic	Orientation
HCN	6	7	140
HCl	8	1.2	22
HI	15	0.7	3
CCl_4	28	3	0

Carbon tetrachloride, being completely symmetrical (as are benzene, methane, etc.), has no dipole moment and shows no effect of orientation. The magnitude of the dipole moment may be taken as a measure of the molecular dissymmetry. Smyth [7] lists the dipole moments of many organic compounds. Since the energy of an electric field is directly proportional to charge and inversely proportional to distance separating charges, on the assumption that a single electron is involved in the dipole moment of an organic molecule, then the charge e would be 4.77×10^{-10} e.s.u. If we take the diameter of a molecule to be approximately 1×10^{-8} cm., the dipole moment of such a molecule would approach 4.77×10^{-18} as an upper limiting value. Most organic molecules have dipole moments somewhat less than this value. They may be thought of as bar magnets, a negative charge residing in one end of the molecule and a corresponding positive charge at distance, δ, toward the other end. When we later discuss molecular orientation we shall make extensive use of this electrical description of molecules.

PRACTICAL METHODS FOR THE PREPARATION OF COLLOIDAL SYSTEMS

As already noted at the beginning of this chapter, the methods of preparation may be divided into (A) Crystallization or Condensation

[7] Smyth, C. P., Dielectric Constant and Molecular Structure, American Chemical Society Monograph 55, The Chemical Catalog Company, New York (1931).

Methods, where we pass from a true solution to colloidal systems; or (B) Solution or Dispersion Methods, where we pass from matter in mass to colloidal micelles.

A. Crystallization or Condensation Methods.—These methods may be divided into six general groups.

1. The Cooling of a Liquid.—Typical examples are water in ether, cooled by carbon dioxide snow, giving rise to a colloidal sol of ice-ether. Referring to equations (1) and (2), we can see that the lowering of the temperature has (*a*) reduced the solubility of the water in the ether, so that the supersaturation $(C - L)$ is much greater, and likewise (*b*) it has reduced the diffusion coefficient (Δ) of the Noyes-Nernst equation and increased the viscosity which will have the same effect as increasing the length of the diffusion path (l). We have thus made for the formation of a greater number of nuclei and have a much slower rate of crystal growth. Accordingly an ice-ether sol is relatively stable at a very low temperature. Other examples could be cited with the same general argument.

2. Replacement of Solvent.—This is a very common method for the formation of colloidal systems, and the success of the method depends again upon factors noted in equations (1) and (2). If we have two solvents, one more or less soluble in the other, one liquid being an excellent solvent for some material which is at the same time practically insoluble in the other liquid, we have conditions where the replacement-of-solvent method can be employed. Thus, sulfur is relatively soluble in carbon disulfide but extremely insoluble in water. If a few drops of a solution of sulfur in carbon disulfide is rapidly stirred into a large volume of water, a beautiful hydrosol of sulfur results. We have here suddenly decreased L and increased the initial values of $(C - L)$, so that many nuclei are formed. The final value of L is so extremely low that almost instantaneously the initial supersaturation $(C - L)$ falls to practically zero, owing to the formation of nuclei. Accordingly when crystal growth takes place $(C - L)$ is essentially zero and therefore the sol is stable for a long period of time.

Sulfur hydrosols and colloidal sulfur are becoming of increasing importance as fungicides. In some instances the sulfur hydrosols are used as sprays; in others the colloidal sulfur has been reduced to dry form, either as the pure sulfur itself or colloidal sulfur associated with carrier material, and is used as a dust.

A very common method for the purification of such materials as gums, proteins, enzymes, etc., is to take advantage of their insolubility in alcohol in order to precipitate them in a form which can be filtered. If one is dealing with a water-soluble protein, it is possible to concentrate it by vacuum evaporation down to a rather viscous sol. If one pours this sol into absolute alcohol, the protein is, as a rule, precipi-

tated in the form of flocs which can be readily gathered and in many instances even filtered off. As the purification progresses, however, and the above process of alcohol precipitation is repeated several times, it frequently happens that no precipitate is formed when the aqueous sol is poured into the alcohol, but instead we have only a limpid, opalescent sol resulting. The problem then arises as to how such a sol can be "broken" so as to cause the formation of the desired precipitate. This can usually be effected by the addition of a small amount of electrolyte (*vide infra*), such as a drop of saturated sodium chloride or preferably lithium chloride. Some sols of this sort are very stable and require a considerable amount of experimentation before the dispersed phase is precipitated.

The beginner in biochemical preparations is probably less likely to lose valuable material when a complete sol results, in an attempted alcohol precipitation, than he is when only a partial sol is formed. It very often happens that a part of the material precipitates in the expected manner, while a greater or a smaller portion remains in the form of a slightly opalescent alcosol. Under such conditions the worker is very likely to decant and discard the supernatant liquor, retaining only the precipitated portion. Many instances have been known where 90 per cent of valuable material went down the drain, as the result of the decantation of supernatant sols. In the preparation of any material by precipitation by pouring into another solvent, it is highly desirable to check the solid content of the supernatant liquor in a qualitative fashion before the supernatant liquor is discarded, in order to be sure that a part of the desired material is not suspended in the form of colloidal micelles.

3. Reduction Methods.—These methods depend upon the reduction of a soluble metallic salt to the insoluble metal. In this group of methods in particular the purity of the water is all-important. Distilled water which has stood for several hours in an ordinary soft-glass bottle usually contains sufficient electrolytes to render the production of the desired sol uncertain. Likewise the purity of the reagents employed becomes a very important factor. Typical examples of colloidal systems formed by the reduction methods are the reduction of auric chloride by formaldehyde, phosphorus, phenylhydrazine, or tannin, as noted by Morrow and Sandstrom[8] or by Holmes.[9] Similarly a beautiful silver sol can be prepared by reduction with hydrogen. If a stream of hydrogen is passed into a hot solution of freshly precipitated silver hydroxide, reduction takes place, and an intensely yellow,

[8] Morrow, C. A., and Sandstrom, W. M., Biochemical Laboratory Methods for Students of the Biological Sciences, Second Edition, pp. 1–5, John Wiley & Sons, New York (1935).

[9] Holmes, H. N., Laboratory Manual of Colloid Chemistry, Third Edition, pp. 33–36, John Wiley & Sons, New York (1934).

essentially electrolyte-free sol of silver results. The reaction which takes place can be expressed as

$$2AgOH + H_2 \rightarrow 2Ag + 2H_2O$$

The sols that are formed by reduction methods are, as a rule, very sensitive to the action of electrolytes and form, from the standpoint of laboratory experimentation, one of the most interesting groups of colloidal systems.

Colloidal metals are gradually coming into use in medicine, and reduction methods appear to be the most suitable for the production of such metallic sols.

4. Oxidation Methods.—Relatively few examples of colloidal systems formed by the use of oxidation processes are recorded. The outstanding example perhaps is the oxidation of hydrogen sulfide and the resulting formation of a sulfur hydrosol.

$$2H_2S + O_2 = 2S + 2H_2O$$

This sol should be familiar to everyone who has ever had any laboratory chemistry. Those who are not interested in colloid phenomena are usually exasperated when they return to the laboratory and find that their solution of hydrogen sulfide has turned to a milky-appearing liquid, and the active hydrogen sulfide has disappeared from the solution. If the bottle in which the hydrogen sulfide solution was stored had been completely filled with the solution and tightly stoppered, this oxidation would not have occurred, but under ordinary laboratory conditions it is an inevitable occurrence. A later stage is the coagulation of the sulfur hydrosol and the precipitation of crystalline sulfur in the bottom of the bottle. Precipitation, however, is rarely complete, and the supernatant liquid over such a sulfur precipitate is usually a dilute sulfur hydrosol. Selenium and tellurium form sols similar to the sulfur hydrosols.

5. Hydrolysis Methods.—Many materials which are readily soluble in water can be made to undergo hydrolysis with the production of an extremely insoluble residue. In such instances appropriate technic will result in the formation of stable sols. Ferric chloride may be chosen as possibly the best example of such a material. The reaction is usually written as

$$FeCl_3 + 3H_2O = Fe(OH)_3 + 3HCl$$

Experiments based on this reaction are given by Morrow and Sandstrom [10] and by Holmes.[11] The above reaction is somewhat mislead-

[10] Morrow, C. A., and Sandstrom, W. M., Biochemical Laboratory Methods for Students of the Biological Sciences, Second Edition, pp. 5–6.

[11] Holmes, H. N., Laboratory Manual of Colloid Chemistry, Third Edition, p. 37.

ing in that it indicates the formation of micelles composed entirely of ferric hydroxide. Weiser and Milligan [12] have shown that those sols which we have normally designated as ferric hydroxide hydrosol and aluminum hydroxide hydrosol are in reality hydrous oxide sols, since the solid phase shows the X-ray diffraction patterns of the oxides. Weiser and Milligan suggest that the composition of the solid phase can best be represented by $[xFe_2O_3 \cdot yHCl \cdot zH_2O]Fe_m^{+++}\ H_n^{+}$ and that these micelles are in ionic equilibrium with chloride ions in the surrounding liquid. We have known for a long time that the micelles in these sols were not pure ferric hydroxide, but it has been believed that they were a mixture of ferric hydroxide and ferric chloride, present in varying ratios. Taylor [13] discusses them under the title, "The 'Complex' Theory of Colloids," and points out that pure ferric "hydroxide" sols, or as a matter of fact most of the lyophobic sols, are very unstable when they are entirely free from admixture with stabilizing ions. Weiser and Milligan's formula indicates that the solid phase is composed of hydrous ferric oxide, ferric chloride, and hydrochloric acid, and that it possesses a positive charge, due to the ionization of the residual ferric chloride and hydrochloric acid. Browne [14] has made studies of the hydrogen-ion concentration and the chloride-ion concentration in such sols and finds that the chloride-ion concentration is more or less proportional to the stability of the sol. Sorum [15] reports a ferric oxide hydrosol which is chloride-free. Weiser and Milligan suggest that this must contain some other electrolyte or some protective material in order to account for its stability.

Hydrolysis methods are applicable to the formation of many hydrosols of the metallic oxides and hydroxides.

6. Double Decomposition or Precipitation Methods.—In many instances the product of the interaction of two soluble materials is an extremely insoluble precipitate. By proper manipulation the precipitate can usually be obtained in the form of a sol. Typical examples are hydrosols of barium sulfate or arsenous sulfide.

$$BaCl_2 + H_2SO_4 = BaSO_4 + 2HCl$$
$$As_2O_3 + 3H_2S = As_2S_3 + 3H_2O$$

[12] Weiser, H. B., and Milligan, W. O., The Constitution of Hydrous Oxide Sols from X-ray Diffraction Studies, *J. Phys. Chem.*, 40: 1–7 (1936), and Colloid Symposium Monograph (1935), pp. 1–7.

[13] Taylor, W. W., The Chemistry of Colloids, p. 108, Longmans, Green and Co., New York (1915).

[14] Browne, F. L., The Constitution of Ferric Oxide Hydrosol from Measurements of the Chlorine- and Hydrogen-ion Activities, *J. Am. Chem. Soc.*, 45: 297–311 (1923).

[15] Sorum, C. H., The Preparation of Chloride-free Colloidal Ferric Oxide from Ferric Chloride, *J. Am. Chem. Soc.*, 50: 1263–1267 (1928).

Probably every analytical chemist has had the undesirable experience of a barium sulfate precipitate going through the filter paper. The conditions of precipitation had been such that a more or less stable hydrosol had been formed. Such conditions are usually brought about when there is a nearly exact equivalence of barium chloride and sulfuric acid and when the resulting concentration of hydrochloric acid is very low. Precipitation at the boiling temperature, and the general practice of allowing a barium sulfate precipitate to stand for a number of hours before filtering, both favor the growth of crystals and the subsequent increase in particle size, so that the precipitate is retained on the filter paper. Following the formation of the nuclei, the elevated temperature increases L the solubility of the disperse phase and $(C - L)$ the absolute supersaturation, at the same time increasing Δ, the diffusion coefficient, and decreasing viscosity which will likewise increase the rate of diffusion. The length of the diffusion path (l) will be decreased, owing to the convection currents and stirring at the higher temperature, so that it may become essentially negligible.

Trimble [16] points out that crystal growth ceases to be a factor when the particles of barium sulfate are larger than 2 μ apparent diameter, but that aggregation and cementing together of the particles is an important factor in the retention of the precipitate on the filter paper.

Kolthoff [17] and his students have been very active in investigating the changes which take place after the initial formation of a precipitate. Space in the present volume is too limited to permit of a discussion of the findings, but the student of analytical chemistry should consult these papers, inasmuch as they have a profound bearing on the purity of analytical precipitates.

In the arsenous sulfide sols, no precipitating ion is formed by the reaction, and extremely stable sols can accordingly be prepared. Here again we are probably not dealing with pure As_2S_3 micelles, but apparently have a complex ion of arsenous sulfide stabilized by the sulfhydril (SH^-) ion, due to the presence of a slight excess of hydrogen sulfide, so that the micelle actually possesses the formula

$$(As_2S_3)_x SH^-$$

It is a common experience for the analytical chemist in precipitating the sulfides of arsenic, antimony, tin, silver, etc., to find that they

[16] Trimble, H. M., The Coalescence of an Unfilterable Precipitate of Barium Sulfate, *J. Phys. Chem.*, 31: 601–606 (1927).

[17] Kolthoff, I. M., *et al.*, Structural Changes Taking Place During the Aging of Freshly Formed Precipitates, *J. Am. Chem. Soc.*, 56: 1264–1269; 1658–1661 (1934); 57: 597–600; 607–611; 2573–2577; 2577–2579 (1935); 58: 116–120; 121–122 (1936); Studies in Coprecipitation, *J. Phys. Chem.*, 36: 549–566; 860–881 (1932); 37: 153–170; 443–458; 459–473; 723–733 (1933).

occasionally are not retained on a filter paper. In such instances hydrogen sulfide has been passed into the solution for too long a period of time and the initial precipitate of sulfide has become stabilized by the adsorption of some of the excess hydrogen sulfide with a resulting hydrosol formation.

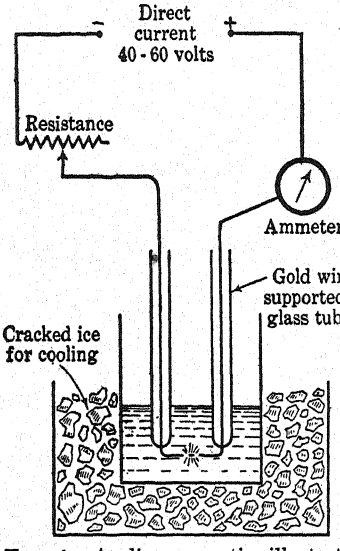


FIG. 4.—A diagrammatic illustration of the Bredig method of preparing metallic sols by electrical pulverization.

B. SOLUTION OR DISPERSION METHODS.—These methods may be divided into two general groups: (1) electrical dispersion, and (2) peptization.

1. Electrical Dispersion.—Bredig,[18] in 1898, announced a general method for the preparation of metallic sols by the process of electrical pulverization. When an arc is passed between two electrodes, one of the electrodes becomes corroded. Bredig made use of this phenomenon by striking an arc between metallic electrodes inserted under the surface of a liquid. Figure 4 is a diagrammatic representation of such an apparatus. Using thick wires of gold, platinum, silver, etc., and a direct current of 4 to 10 amperes at 35 to 50 volts, he was able to prepare metallic sols not only in water as a dispersions medium but also in certain of the organic solvents, although the latter offer difficulties, owing to the separation of colloidal carbon by the decomposition of the organic liquid in the heat of the electric arc.

When the points of two gold wires are momentarily brought together beneath the surface of the water and then separated, an arc is struck between the points. The tip of the cathode becomes molten, and a stream of fine gold particles is shot from the molten cathode toward the anode. In the heat of the arc and under the influence of the electric current some of the smaller of these particles may vaporize or become re-subdivided; in either event part of the gold fails to reach the anode, instead being dispersed in a colloidal cloud which rises through the liquid from the vicinity of the arc with the appearance of a rising cloud of smoke or dust. The gold particles which do reach the anode fuse on its tip, so that the wire forming the anode lengthens

[18] Bredig, G., Darstellung colloidaler Metallösungen durch elektrische Zerstäubung *Z. angew. Chem.*, 1898, pp. 951–954.

as the wire forming the cathode shortens. A certain amount of coarse, granular gold dust, derived from larger particles of gold which failed to reach the anode, is deposited in the bottom of the beaker. It is preferable to bring the electrodes in contact and later to separate them so as to strike the arc, using a mechanical device of springs and set screws, rather than to attempt to operate the electrodes by hand. Kraemer and Svedberg [19] modified Bredig's original method, making use of a high-frequency alternating current arc instead of a direct current, and by use of this modified technic prepared metallic organosols containing minimal amounts of carbon. Svedberg [20, 21] had earlier used an induction coil for the preparation of similar sols.

Both vaporized metal and finely divided molten metal contribute to the Bredig sols. Such sols contain a rather wide range of particle size, the sizes varying from particles which gradually settle out, down to particles near the lower limit of the colloid realm. The range in particle size in sols obtained by the Bredig method is much greater than it is in metallic sols obtained by the reduction methods, and as a general rule it is easier to obtain sols with small-sized particles (10–25 mμ) using reduction methods, than it is to obtain similar sols by the Bredig method. The noble metals rather readily yield sols by the Bredig method. Using other metals as electrodes the sols which are obtained are usually contaminated with larger or smaller amounts of oxides and hydroxides.

Bredig's method offers an extremely valuable technic for fundamental studies of the properties of colloid systems, since sols obtained from pure metallic electrodes and conductivity water contain only metallic particles suspended in a pure liquid and as a result the influence of minute traces of electrolytes on colloid stability can be investigated. Burton [22] has made extensive observations of the behavior of metallic sols, particularly silver sols prepared by Bredig's method. For a more extended discussion of the Bredig sols, the reader is referred to Freundlich [23] and Svedberg.[24]

2. Ultrasonic Dispersion.—The use of ultrasonic waves is a recent

[19] Kraemer, E. O., and Svedberg, The, Formation of Colloid Solutions by Electrical Pulverization in the High-Frequency Alternating Current Arc, *J. Am. Chem. Soc.*, 46: 1980–1991 (1924).

[20] Svedberg, The, Über die elektrische Darstellung einiger neuen colloidalen Metalle, *Ber.*, 38: 3616–3620 (1905).

[21] Svedberg, The, Über die elektrische Darstellung colloidaler Lösungen, *Ber.*, 39: 1705–1714 (1906).

[22] Burton, E. F., On the Properties of Electrically Prepared Colloidal Solutions, *Phil. Mag.*, (6) 11: 425–447 (1906).

[23] Freundlich, Herbert, Colloid and Capillary Chemistry, pp. 509–513. Translated from the German by H. S. Hatfield. E. P. Dutton and Company, New York (1926).

[24] Svedberg, The, Colloid Chemistry, Second Edition, The Chemical Catalog Company, New York (1928).

method for bringing about colloidal dispersion. Wood and Loomis [25] appear to be the first workers to have utilized this technic. The effect of ultrasonic waves in the preparation of colloidal systems and the influence of ultrasonic waves on colloidal systems have been extensively investigated in Freundlich's laboratory.[26–36]

A piezoelectric quartz plate is set into vibration by a high-frequency current. The waves so produced are in reality sound waves vibrating above the limit of audibility in the frequency range of approximately 200,000 cycles per second. The disturbances set up in the liquid cause fragmentation of gross particles suspended in the liquid with the production of particles of colloidal size. Here again it is possible by this technic to prepare sols in the essential absence of electrolytes. Ultrasonic waves fragment bacteria and destroy animal life in the liquid subjected to the vibration.

3. Peptization.—Peptization is undoubtedly the most important of all dispersion methods. Graham,[37] in 1864, coined the term designating this phenomenon. Graham says, "The solution of these colloids, in such circumstances, may be looked upon as analogous to the solution of insoluble organic colloids witnessed in animal digestion. . . . Liquid silicic acid may be represented as the 'peptone' of gelatinous silicic acid; and the liquefaction of the latter by a trace of alkali, may be spoken of as the peptization of the jelly."

[25] Wood, R. W., and Loomis, A. L., Physical and Biological Effects of High-frequency Sound Waves of Great Intensity, *Phil. Mag.*, (7) 4: 417–436 (1927).

[26] Freundlich, H., Rogowski, F., and Söllner, K., Über die Wirkung der Ultraschallwellen auf thixotrope Gele, *Z. physik. Chem.*, Abt. A, 160: 469–472 (1932).

[27] Freundlich, H., Söllner, K., and Rogowski, F., Einige biologische Wirkungen von Ultraschallwellen, *Klin. Wochenschr.*, 11 (II): 1512–1513 (1932).

[28] Bull, H. B., and Söllner, K., Über Quecksilberemulsionen, die mit Hilfe von Ultraschallwellen hergestellt wurden, *Kolloid Z.*, 60: 263–268 (1932).

[29] Freundlich, H., Rogowski, F., and Söllner, K., Die Einwirkung von Ultraschallwellen auf Gele, insbesondere thixotrope Gallerten, *Kolloid Beihefte,* 37: 223–241 (1933).

[30] Bondy, C., and Söllner, K., On the Mechanism of Emulsification by Ultrasonic Waves, *Trans. Faraday Soc.*, 31: 835–842 (1935).

[31] Bondy, C., and Söllner, K., The Influence of Gases on Mercury Emulsions Prepared by Ultrasonic Waves, *Trans. Faraday Soc.*, 31: 843–846 (1935).

[32] Bondy, C., and Söllner, K., Quantitative Experiments on Emulsification by Ultrasonic Waves, *Trans. Faraday Soc.*, 32: 556–567 (1936).

[33] Söllner, K., and Bondy, C., The Mechanism of Coagulation by Ultrasonic Waves, *Trans. Faraday Soc.*, 32: 616–623 (1936).

[34] Freundlich, H., and Söllner, K., The Influence of Ultrasonic Waves on Gels, *Trans. Faraday Soc.*, 32: 966–970 (1936).

[35] Söllner, K., The Mechanism of the Formation of Fogs by Ultrasonic Waves, *Trans. Faraday Soc.*, 32: 1532–1536 (1936).

[36] Söllner, K., Experiments to Demonstrate Cavitation Caused by Ultrasonic Waves, *Trans. Faraday Soc.*, 32: 1537–1539 (1936).

[37] Graham, Thomas, On the Properties of Silicic Acid and Other Analogous Colloidal Substances, *J. Chem. Soc.*, 17: 318–327 (1864).

The older statements were to the effect that the peptizer must (*a*) have one ion in common with the material to be dispersed, or (*b*) be capable of forming a soluble compound with the material to be dispersed, or (*c*) have one ion which is very strongly adsorbed by the material being dispersed. Kruyt [38] has clarified these statements by pointing out the actual conditions which must be met. His viewpoint is that lattice forces are responsible for the inner coat of the double layer, the silver halides being a model example. With AgI, the I ions from KI fit into the open spots of the lattice surface, or Ag^+, if $AgNO_3$ is used as a peptizer. "Although every ion with an isomorphic relation to those in the lattice is able to peptize, no other ion whatever can accomplish this. Other ions may increase the potential of the double layer, but they are not able to form the original double layer." An AgS sol formed by peptization with H_2S is enormously stabilized by $K_4Fe(CN)_6$ by reason of an increased *zeta*-potential, but $K_4Fe(CN)_6$ is not able to peptize HgS; only H_2S or SH^- can do this, since these ions can distribute themselves in the lattice as well as in the surrounding liquid medium.

We have already noted the "complex theory of colloids," using the example of a ferric oxide hydrosol where the micelle probably has the formula of $(Fe_2O_3 \cdot nH_2O)_x(FeCl_3)_y^+$. In this case the ferric chloride can be regarded as the peptizing agent of the hydrous ferric oxide. If precipitated hydrous ferric oxide is suspended in water and heated to near the boiling point (the heat is merely to hasten the reaction) and a small amount of hydrochloric acid added, it will be found that within a short time the precipitated flocculent hydrous ferric oxide has dispersed to a transparent yellow-brown sol which will readily pass through a filter paper. The amount of hydrochloric acid which is necessary to bring about such a peptization may be less than 1 per cent of the amount which would be necessary to convert into ferric chloride the hydrous ferric oxide which is present. The small amount of ferric chloride which is formed by this reaction acts as a peptizing agent on the flocculent hydrous ferric oxide, dispersing the flocs into smaller aggregates of colloidal dimensions and stabilizing these aggregates probably by adsorption of a small amount of the ferric chloride.

A similar sol can be made by the peptization of hydrous aluminum oxide with hydrochloric acid. Peptization can likewise be induced by adding ferric chloride to hydrous ferric oxide or aluminum chloride to hydrous aluminum oxide. Even ammonium hydroxide may act as a peptizing agent, and it is for this reason that texts in analytical chemistry stress the fact that the ammonia used in precipitating the R_2O_3 group of metals must be boiled off before the precipitate is filtered. Other-

[38] Kruyt, H. R., The Modern Development of Colloid Chemistry, *Act. IX Congr. Intern. quím. pura y aplicada,* Madrid, April, 1934 (separate 12 pp.).

wise a certain amount of hydrous aluminum oxide sol may pass through the filter paper, and a loss of aluminum result.

Hydrogen sulfide is a very efficient peptizing agent for sulfides. In the case of the arsenous sulfide sols, the reaction probably is

$$As_2S_3 + H_2S \rightarrow (As_2S_3)SH^-$$

It should be stressed that such micelles as

$(As_2S_3)SH^-$, $(Fe_2O_3 \cdot nH_2O)_xFe^{+++}$, or $(Fe_2O_3 \cdot nH_2O)_x(FeCl_2)_y^+$, etc.,

are not chemical compounds in the sense that they represent a pure, chemical individual. Their composition is not constant but varies continuously through a wide range, depending on the amount of peptizing agent which is adsorbed.

The analytical chemist who ignores the phenomena of peptization is very likely to secure results which are seriously in error, for many of the precipitates of gravimetric analyses are readily peptized when in a moist condition. Figure 5 illustrates diagrammatically some general phenomena applicable to the gravimetric determination of silver

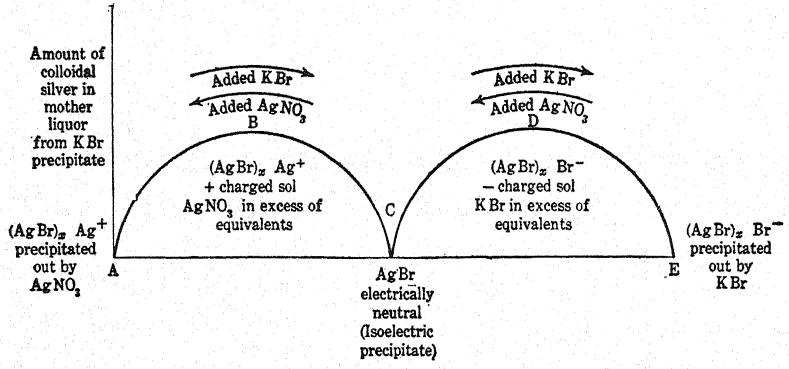


FIG. 5.—A diagrammatic illustration of peptization, the influence of ions on the sign of the charge on the micelle, isoelectric precipitation, and "salting out" at high salt concentrations.

as silver bromide and to the peptization of precipitated silver bromide to form stable silver bromide sols. If exactly equivalent solutions of silver nitrate and potassium bromide are mixed, an isoelectric precipitate of silver bromide will be formed, as shown at the point *C* on the diagram. *This is the only point on the diagram where strictly pure silver bromide is found and where the weight of the precipitate accurately represents the amount of silver present in the solution.* The precipitates which are obtained at the points *A* and *E* will be heavier than the precipitate obtained at *C*, and accordingly will give high results for silver and bromine respectively, whereas precipitates obtained

anywhere along the curve *AC* will be light in weight and give low results for bromine, or along the curve *CE* will give low results for silver. Anyone who is interested in a consideration of the nicety of technic necessary for the *accurate* determination of silver should read some paper dealing with the determination of atomic weights where silver is used as the reference standard. It is in such determinations that extreme accuracy is necessary, and in such experiments the precipitation of isoelectric silver bromide or chloride is rigidly controlled.

If one adds an excess of potassium bromide to the precipitated silver bromide, the amount of silver in the mother liquor follows the curve *CDE*, the point reached depending on the amount of potassium bromide added in excess. Throughout the entire area under the curve *CDE* there is formed a precipitate of silver bromide containing adsorbed bromine ions and the supernatant liquid is a negatively charged silver bromine sol, probably of the composition $(AgBr)_xBr^-$. At point *D* on the curve we attain the greatest concentration of the negatively charged silver bromide micelles, a further addition of potassium bromide causing precipitation of the colloidal particles, so that finally at *E* there is no silver in the mother liquor and a precipitate forms which appears to be silver bromide but in reality is silver bromide containing a considerable excess of bromine, probably contaminated with potassium bromide.

A similar argument can be made for the region lying under curve *ABC* except that in this instance silver nitrate is added in excess. Throughout this area we have a positively charged sol in the mother liquor, the composition of the micelles being represented as $(AgBr)_xAg^+$. This sol reaches its maximum concentration at point *B* and is precipitated by the excess silver nitrate at point *A*, the precipitate having the composition noted on the diagram. The above example has been cited in detail because it is typical of the behavior of many precipitates met with in the various fields of chemistry.

The change in the sign of the charge on the silver precipitate with excess halogen ions or excess silver ions has been made use of by Kolthoff, Lauer, and Sunde,[39] who use dichlorofluorescein as an indicator in the titration of chlorides with silver nitrate. As long as the precipitate is negatively charged (an excess of Cl^- is present), there is no adsorption of the dyestuff by the precipitate. The instant that there is an excess of Ag^+ in the solution, the colorless silver chloride becomes positively charged and adsorbs the anion of the dyestuff, the colorless precipitate instantly becoming an intense red. The advantage of this indicator over the usual chromate indicator is that it can be used even in solutions which are decidedly acidic.

[39] Kolthoff, I. M., Lauer, W. M., and Sunde, C. J., The Use of Dichlorofluorescein as an Adsorption Indicator for the Argentometric Titration of Chlorides, *J. Am. Chem. Soc.*, 51: 3273–3277 (1929).

The peptization of matter in mass may be brought about in many ways, chemically, mechanically, and electrically. In each instance, however, *energy must be added to the system.* As we shall see when we consider surface phenomena (*vide infra*), a given mass of material dispersed in small micelles has a much greater energy content than the same mass of material when present in large aggregates. This energy must be added to the system in some form if we are to peptize matter in mass.

Peptizing methods fall into four general classes.

a. The Washing Out of a Precipitating Agent.—Everyone familiar with quantitative analysis recognizes the fact that certain precipitates cannot be washed with pure water without causing the precipitate to go through the filter paper. Thus, we wash the "yellow precipitate" of ammonium phosphomolybdate with a dilute solution of ammonium nitrate rather than with pure water. The ammonium nitrate prevents the precipitated ammonium phosphomolybdate from dispersing into a colloidal sol. Similarly, if we were to wash the precipitate of silver bromide obtained at points *A*, *C*, *E* on the diagram in Fig. 5, we would find that the curves *ABC* and *CDE* were reversible and that, as the silver nitrate or potassium bromide was washed out of precipitates *A* and *E*, the liquid passing through the filter would contain silver bromide micelles positively charged in the case of precipitate *A* and negatively charged in the case of precipitate *E*. The isoelectric precipitate obtained at point *C* would not be peptized by the action of water, and *such isoelectric material is the only material which would give accurate information in regard to the solubility of silver bromide in water.* In the great majority of instances where a precipitate goes through the filter paper we are dealing with the peptization of a precipitated colloid system (a gel or a coagulum) by the washing out of the precipitating agent.

In the mechanical analysis of soils this phenomenon comes into play. If a soil is leached with hydrochloric acid and then washed free of chlorides with distilled water, it is the general experience that before the chlorides are completely removed clay begins to pass through the filter paper. The clay in the soil is usually present in a more or less precipitated condition, due to the presence of inorganic salts. The removal of these salts permits the peptization of the clay aggregates to take place with the formation of a clay hydrosol. If the soil be a surface soil, the black humus likewise becomes peptized by washing out the calcium or other metallic salts which hold it in a precipitated condition, and it often happens that appreciable amounts of humus can be extracted from the soil in the form of a colloidal sol by first leaching the soil with hydrochloric acid to remove the calcium, followed by washing with distilled water to the absence of chlorides.

Many organic substances are peptized by the direct addition of

water. Familiar examples are gelatin, gum arabic, and dextrin. In certain instances it is necessary to add energy in the form of heat to cause complete dispersion. Thus, agar and gelatin form gels in cold water, but sols in hot water.

b. The Addition of a Peptizing Agent.—This has already been discussed fully in the sections above. Specific examples are: the addition of ammonium hydroxide to a soil in order to form a clay hydrosol; the peptization of china clays by alkalies or carbon dioxide; the removing of dirt from clothing by soap; stable sols of lamp black and graphite by the use of soaps, proteins, gums, and resins; emulsions, such as kerosene emulsion, so widely used in spraying, by the use of soaps. A great number of laboratory or technical applications have been made [40] involving peptization of one or another material by the addition of a peptizing agent.

c. Mechanical Disintegration.—As noted above, peptization occurs whenever energy is added to a system. This energy may be added in the form of mechanical energy, *i.e.*, grinding. If one places a fragment of a microscope cover glass in a mortar and retains sufficient enthusiasm to grind this fragment vigorously for 20 to 30 minutes, it will be found that a considerable part of the glass will remain as a stable hydrosol when water is added to the ground mass. Similarly, almost any practically insoluble material can be obtained in the form of a colloidal sol. Most insoluble materials form lyophobic sols. The formation of lyophobic sols requires a much greater expenditure of mechanical energy than does the formation of similar lyophilic sols. Most lyophilic sols form more or less spontaneously on the addition of the liquid.

Colloid mills have been devised to prepare finely divided materials for industrial uses. Most of these mills are misnamed "colloid" mills, inasmuch as the size of the suspended particle rarely falls within the limits fixed by the colloid realm. On the other hand, the disintegration produced by such mills is greater than that generally produced by other grinding methods, and such apparatus is coming more and more into use for the grinding of pigments, the making of emulsions or finely dispersing any desired material in a liquid.

d. Electrical Energy.—The formation of colloidal systems by the use of electrical energy may be regarded as a form of peptization. We have already discussed it under electrical dispersion, and it is referred to again, inasmuch as some authors make no distinction between electrical dispersion and peptization. Whether or not such a distinction

[40] Compare, for example, Bancroft, W. D., Report on Peptization and Precipitation, Second Report on Colloid Chemistry, Brit. Assoc. for the Advancement of Science (1918); Bogue, R. H., The Theory and Application of Colloidal Behavior, Vol. II, McGraw-Hill Book Company, New York (1924); and Fall, P. H., Detergent Action of Soaps, *J. Phys. Chem.*, 31: 801–849 (1927).

is made will depend upon how closely lines are drawn in a definition.

EMULSIONS.—Possibly emulsions could be classified as a fifth group of colloidal systems formed by peptization. However, the range of particle size in emulsions generally exceeds the upper limit of the colloid realm, so that many of the fat droplets in emulsions are visible in the ordinary microscope field.

An emulsion, in its simplest form, can be defined as a mixture of two mutually insoluble liquids. It is obvious from this definition that *two different types of emulsions are possible, depending upon which liquid forms the disperse phase and which liquid forms the dispersions medium.* When water is one of the components we distinguish these two forms of emulsions by oil-in-water or water-in-oil emulsions. Emulsions which are met with in biological phenomena almost invariably have water as one of the phases.

It is extremely difficult to produce simple emulsions, *i.e.*, emulsions containing only one pure liquid dispersed in another pure liquid, which have any appreciable concentration and which are stable. In certain instances dilute emulsions (1:1,000) can be obtained where the above conditions are met. For example, in the preparation of aniline by steam distillation, the supernatant liquid in the receiving flask very often is a milky appearing liquid, a dilute emulsion of aniline in water. Such emulsions are often obtained in the distillation of volatile liquids which are relatively insoluble in water. If the bulk of such emulsions is small, this phenomenon does not constitute a serious loss in industrial processes unless the suspended material is extremely valuable. With certain essential oils, the material remaining in the emulsion must be recovered either by extracting with a solvent or by breaking the emulsion in some manner, causing the oil to separate.

Most emulsions used in the industries are stabilized by the presence of another disperse system, preferably a colloid which shows a marked affinity for either the oil or the water. Gums are the substances most generally used as stabilizers. Gum acacia (gum arabic) is the common stabilizer for oil-in-water emulsions, although proteins act similarly. Probably the reason that the emulsions used in the industries are stabilized with gum acacia instead of proteins is that proteins are readily attacked by putrefactive bacteria, whereas gum acacia is not. Gum acacia disperses in water to form a lyophilic hydrosol. It is a general rule that *those emulsion stabilizers which are soluble in water or which form lyophilic hydrosols produce emulsions of the oil-in-water type, whereas those stabilizers which are insoluble in water and soluble in oil produce emulsions of the water-in-oil type.* The sodium or potassium soaps, being water-soluble, act as stabilizers of oil-in-water emulsions, whereas the calcium soaps, being water-insoluble and oil-soluble, serve as efficient stabilizers for water-in-oil emulsions. Gum

dammar, an oil-soluble gum, is an efficient stabilizer of water-in-oil emulsions.[41]

As a rule the natural emulsions, such as milk and the milky latex of plants, are stabilized by proteins. The latex of certain of the *Euphorbiaceae* is stabilized by sterols and in other instances by proteins.[42] The rubber hydrocarbon in *Hevea brasiliensis* is held in the latex by a protein which has more or less the characteristics of a globulin. A special protein [43-45] stabilizes the fat droplets in milk and cream. The egg-yolk proteins stabilize mayonnaise dressing, and a little gelatin, or better still gum dammar, will make a very stable French dressing emulsion from olive oil and vinegar.

There is apparently no limit to the amount of the disperse phase which may be present in an emulsion. *Ninety-nine per cent of oil can be dispersed in 1 per cent of water containing an emulsifying agent to form an oil-in-water emulsion.* [46, 47] The liquid droplets apparently pack and deform into a more or less honeycomb structure with only a thin film of water between them. Obviously such emulsions possess a certain degree of rigidity or a rather high viscosity. It is doubtful that such concentrated emulsions could be formed without a distortion of the fat droplets, for a mixture of such concentration could not be obtained in the case of a solid dispersed in water. For example, lead shot and water reach a limit of shot content depending on the size of the shot.

Pickering points out that various solid materials, such as barium sulfate, precipitated sulfur, Prussian blue, Trumbull's blue, and purple of Cassius can be used to stabilize emulsions. In such instances the precipitate has an affinity for either the water or the oil and collects on the surface of the droplet, forming a more or less rigid membrane which prevents a later coalescence of the disperse phase. Schlaepfer [48]

[41] Holmes, H. N., and Cameron, D. H., Gum Dammar as an Emulsifying Agent, *Science*, 56, 724 (1922); Emulsion, U. S. Patent 1,429,430, dated Sept. 19, 1922.

[42] Moyer, L. S., On the Surface Composition of Certain Latex Particles, *Am. J. Bot.*, 22: 609–627 (1935).

[43] Palmer, L. S., and Wiese, Hilda F., Substances Adsorbed on the Fat Globules in Cream and their Relation to Churning. II. The Isolation and Identification of Adsorbed Substances, *J. Dairy Sci.*, 16: 41–57 (1933).

[44] Wiese, Hilda F., and Palmer, L. S., Substances Adsorbed on the Fat Globules in Cream and their Relation to Churning. III. Analysis of the Adsorbed Protein, *J. Dairy Sci.*, 17: 29–32 (1934).

[45] Rimpila, C. E., and Palmer, L. S., Substances Adsorbed on the Fat Globules in Cream and their Relation to Churning. IV. Factors Influencing the Composition of the Adsorption "Membrane," *J. Dairy Sci.*, 18: 827–839 (1935).

[46] Pickering, S. U., Über Emulsionen, *Kolloid Z.*, 7: 11–16 (1910).

[47] Pickering, S. U., Emulsions, *J. Chem. Soc.*, 91: 2001–2021 (1907).

[48] Schlaepfer, A. U. M., Water-in-oil Emulsions, *J. Chem. Soc.*, 113: 522–526 (1918).

concluded that a finely divided solid which was more easily wetted by oil than by water should be capable of forming emulsions with water as a disperse phase. He used lamp black in order to test out his theory and formed stable water-in-oil emulsions. Talmud and Suchowolskaja [49] discuss three-phase emulsions of oil-water-insoluble powders with reference to emulsification, gelation, and syneresis. They do not believe that electrical charges play any appreciable role but that solvation is the important factor and that this in turn is due to the wettability of the solid phase, *i.e.*, the presence of polar or non-polar groups. They suggest that syneresis occurs when polar groups disappear through combination with some other surface and that consequently the ability to hold the dispersions medium is lost. Berl and Schmitt [50] have adopted this view and designate a powder as hydrophilic, *i.e.*, it is preferentially wetted by water, if it stabilizes an oil-in-water emulsion, and as hydrophobic if it stabilizes a water-in-oil emulsion, and apply the results of their investigation to the problems of ore flotation.

We have already indicated that ultrasonic waves may produce emulsions. Bondy and Söllner (*loc. cit.*) investigated the mechanism of this emulsification and conclude that it is due to cavitation (collapse of gas droplets). In a completely degassed system (*in vacuo*), toluene-water-sodium oleate does not emulsify. Cavitation is always accompanied by a partial degassing and may be produced by streaming liquids under pressure through a small orifice to a low-pressure side. In this way emulsions are easily formed. Too much dissolved gas may hinder emulsion formation owing to cushioning or "buffer" effect, but gases are the real emulsifying agents and are essential to produce the disruption of the liquid. Bondy and Söllner point out that the pressure of cavitation may be enormous, "thousands of atmospheres," and that this becomes of importance in the design of propellers on high-speed torpedo boat destroyers, so much so that, if the propeller is improperly placed, a hole may be worn through the armor plate in a very few days' run.

Bancroft [51] long ago pointed out that in emulsions we are really dealing with two interfacial tensions, one on the water side of the interfacial film and another on the oil side. If the emulsifying agent lowers the interfacial tension on the oil side more than on the water side of the interface, there will be a tendency of the interface to curve

[49] Talmud, D., and Suchowolskaja, S., Modelle lyophiler Kolloide, *Kolloid Z.*, 55: 48–64 (1931).

[50] Berl, E., and Schmitt, B., Über Benetzungsversuche an hydrophilen und hydrophoben Pulvern im System zweier miteinander nicht mischbarer Flüssigkeiten, I., *Kolloid Z.*, 61: 80–90 (1932).

[51] Bancroft, W. D., Applied Colloid Chemistry, McGraw-Hill Book Company, New York (1921).

and become convex toward the oil side and a water-in-oil emulsion will result, whereas the reverse interfacial tension effects will cause the film to be concave toward the oil side and favor an oil-in-water emulsion.

Roberts [52] expanded Bancroft's theory of a double interface and discussed the energy relations which exist on the two interfaces with respect to the adsorption of polar and non-polar molecules, the adsorption of ions, and the electrical double layers which may arise from such adsorption affinities. He points out that, by *pulling* the ring from a du Noüy surface tension balance through the surface, different interfacial tensions can be detected from those obtained when the ring is *pushed* through the surface from the opposite side, and he has applied his theory, with gratifying results, to the problems encountered in natural crude oil emulsions.

The double interfacial tension theory is probably the best theory that we have at present to account for emulsion formation and stability, and emulsion behavior will probably be better understood when we have a large series of data of the interfacial tension forces on the two sides of the stabilizing layer. Unfortunately such data are difficult to obtain and quantitatively are practically non-existent, so that for the present we must be content with the usual single interfacial tension measurements.

Emulsions are stable when the interfacial tension between the phases is low, and *any substance which greatly lowers the interfacial tension of water will tend to stabilize an emulsion of which water is one component.* Donnan and Potts [53] have shown that the first fatty acid to produce an emulsifying effect is lauric acid (C_{12}), and that this is the first one greatly to lower the surface tension of water at low concentrations of the sodium salt. Table III, calculated from their data, shows the percentage lowering of the interfacial tension between pure paraffin oil and water, when relatively small quantities of the sodium salts of a homologous series of the saturated aliphatic acids are added to the aqueous phase, and Fig. 6 is a graphic representation of the experimental data.

Finkle, Draper, and Hildebrand [54] and Harkins [55] are undoubtedly correct in ascribing the action of efficient emulsifiers to the collecting of

[52] Roberts, C. H. M., A New Theory of Emulsions, *J. Phys. Chem.*, 36: 3087–3107 (1932); Colloid Symposium Monograph, Vol. X, pp. 239–259 (1932).

[53] Donnan, F. G., and Potts, H. E., Ueber die Emulgierung von Kohlenwasserstoffölen durch wässerige Lösungen fettsaurer Salze, *Kolloid Z.*, 7: 208–214 (1910).

[54] Finkle, P., Draper, H. D., and Hildebrand, J. H., The Theory of Emulsification, Colloid Symposium Monograph, Vol. I, pp. 196–209 (Published by the Department of Chemistry, University of Wisconsin, Madison, 1923).

[55] Harkins, W. D., The Orientation of Molecules in the Surfaces of Liquids, Colloid Symposium Monograph, Vol. II, pp. 141–173, Chemical Catalog Company, Inc., New York (1925).

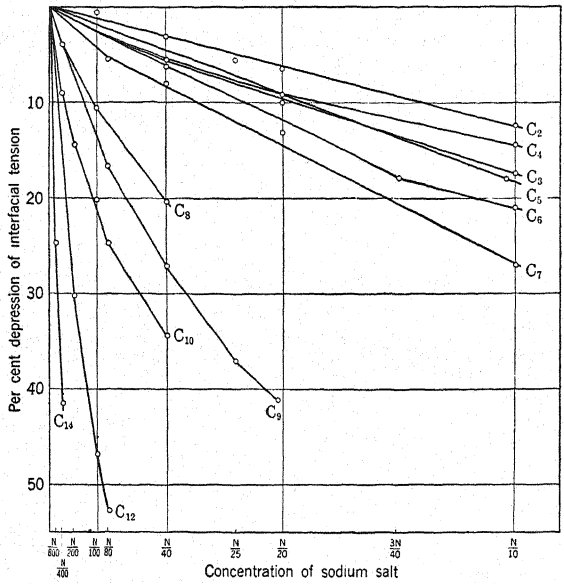


FIG. 6.—The effect of the sodium salts of homologous series of fatty acids on the lowering of the interfacial tension of a water-paraffin oil system. (Data of Donnan and Potts.)

TABLE III

THE PERCENTAGE LOWERING OF THE INTERFACIAL TENSION BETWEEN PURE PARAFFIN OIL AND WATER, DUE TO THE ADDITION TO THE AQUEOUS PHASE OF SMALL QUANTITIES OF THE SODIUM SALTS OF A HOMOLOGOUS SERIES OF THE SATURATED ALIPHATIC ACIDS

Sodium Salt of	Number of Carbon Atoms	Lowering of Interfacial Tension	
		Concentration, $\frac{n}{400}$	Concentration, $\frac{n}{100}$
		Per Cent	Per Cent
Acetic acid	2	0.3	0.2
Propionic acid	3	0.6	2.3
Butyric acid	4	0.6	2.3
Valeric acid	5	0.5	2.0
Caproic acid	6	0.6	2.4
Oenanthylic acid	7	1.0	3.6
Caprylic acid	8	3.5	10.6
Pelargonic acid	9	4.1	13.8
Capric acid	10	9.2	20.4
Lauric acid	12	21.6	46.2
Myristic acid	14	41.0	....

the emulsifying agent in the interfacial film in such a manner that the oil droplets are surrounded by a "shell" of oriented molecules of the stabilizing agent. The theory which Finkle, Draper, and Hildebrand propose to explain the formation of oil-in-water emulsions when the alkali salts of the fatty acids are used, and the formation of water-in-oil emulsions when the fatty acid salts of the alkaline earths or of the heavy metals are employed, is incorrect, inasmuch as the size of a molecule of a stabilizing soap is not sufficiently great to exert the leverage action which is postulated; *cf.* Harkins.[56] On the other hand, there is no doubt but that, in emulsions stabilized by a soap, the hydrocarbon end of the molecule of the salt of a fatty acid is oriented toward the oil phase and the carbonyl group with its metallic radical is oriented toward the water phase. Figure 7 shows diagrammatically such orientation. In all probability the explanation of the change in form of the curves in Fig. 6 can be accounted for on the theories developed by Harkins,[57] and Langmuir [58] dealing with the orientation of molecules in surface films.

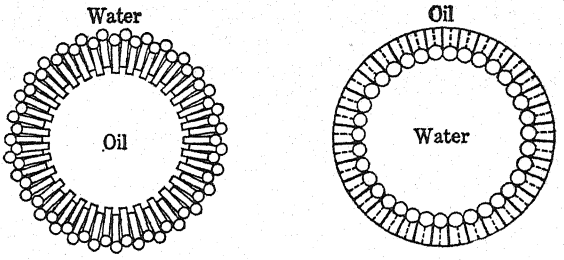


FIG. 7.—A diagrammatic representation of molecular orientation of the stabilizing molecules in oil-in-water and water-in-oil emulsions. (After Harkins.)

This question of molecular orientation will be more fully considered in a later chapter. It is sufficient to note at this point that the suggested explanation for the progressive reduction of surface tension in aqueous solutions of the alkali salts of the fatty acids with progressive increase in the length of the carbon chain is accounted for by the eventual formation of a hydrocarbon interface between liquid and air. When such an interface is complete and we have a continuous, closely packed "skin" of oriented molecules on the surface of the water, we no

[56] Harkins, W. D., Colloid Symposium Monograph, Vol. II, pp. 141–173, Chemical Catalog Company, Inc., New York (1925).

[57] Harkins, W. D., Colloid Symposium Monograph, Vol. II, pp. 141–173 (1925); Surface Energy and Surface Tension, Chap. VIII of Colloid Chemistry, Theoretical and Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, New York (1926).

[58] Langmuir, Irving, The Effects of Molecular Dissymmetry on Some Properties of Matter, Chap. XXIX of Colloid Chemistry, Theoretical and Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, New York (1926); The Distribution and Orientation of Molecules, Colloid Symposium Monograph, Vol. III, pp. 48–75, Chemical Catalog Company, New York (1925).

longer have a water-air interface but rather a hydrocarbon-air interface which, as will be noted by reference to Table III, has a much lower surface tension than a water-air interface.

As we increase the length of the carbon chain past C_{14}, there is relatively little change in the effect on surface tensions or interfacial tension. This is to be expected, inasmuch as if we are already dealing with a hydrocarbon surface, the addition of a $—CH_2—$ radical underneath that surface should have but little effect on the properties of the surface.

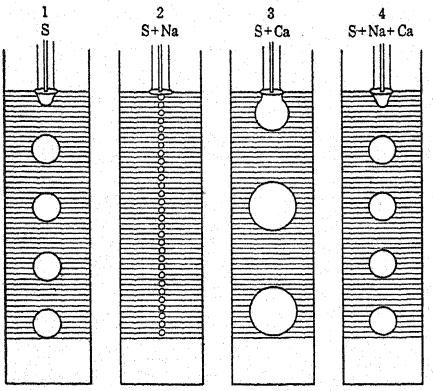


FIG. 8.—Illustrating Expts. 1–4 in Table IV. In each case the number of drops in the diagram is one-tenth of the number actually obtained. (After Clowes.)

Clowes [59] studied the effect of sodium chloride and calcium chloride on emulsification. Figure 8 and Table IV, taken from his data, represent the experimental results. The experimental technic was to allow slightly rancid olive oil to flow from the tip of a stalagmometer and to count the number of droplets which are formed by a given volume of the oil. It will be noted that sodium chloride solutions greatly decrease interfacial tension with the resulting formation of small droplets, whereas calcium chloride solutions increase interfacial tension with the resulting formation of large droplets. The action of the sodium chloride and calcium chloride in the above experiments is explained by the formation of small amounts of sodium or calcium soaps, and though no emulsion resulted in the above case, it is obvious that the sodium salt increases the ease of dispersion of the olive oil in water, whereas the calcium salt would hinder dispersion.

It is of interest to note that sodium and calcium chlorides when mixed antagonize each other and nullify the effect of either, so that such solutions are without any great effect on interfacial tension. This phenomenon has become known in biological reactions as *antagonism of ions*. Thus, calcium antagonizes sodium, magnesium antagonizes calcium, and potassium antagonizes sodium. Whether or not all these mutually neutralizing effects are due to interfacial tension changes has not been experimentally proved. However, Harkins and Zoll-

[59] Clowes, G. H. A., Protoplasmic Equilibrium. I. Action of Antagonistic Electrolytes on Emulsions and Living Cells, *J. Phys. Chem.*, 20: 407–451 (1916).

TABLE IV

NUMBER OF DROPS FORMED FROM A GIVEN VOLUME OF OLIVE OIL FLOWING FROM A STANDARD TIP INTO SOLUTIONS OF 0.001 *M* NaOH ALONE AND PLUS VARIOUS CONCENTRATIONS OF NaCl AND $CaCl_2$ SOLUTIONS *

Exp. No.	Concentration of			Number of Drops	Interfacial Tension	
	NaOH	NaCl	$CaCl_2$		Olive Oil Ergs for Each Sq. Cm.†	Paraffin Oil Ergs for Each Sq. Cm.†
1	0.001 *M*	.....	.....	44	7.3	7.2
2	0.001 *M*	0.15 *M*	.....	300	0.002	0.00
3	0.001 *M*	.....	0.0015 *M*	24	9.88	9.65
4	0.001 *M*	0.15 *M*	0.0015 *M*	44	6.88	7.48
5	0.001 *M*	0.30 *M*	0.003 *M*	43	6.36	7.12
6	0.001 *M*	0.45 *M*	0.005 *M* ‡	43	6.70	7.36
7	0.001 *M*	0.60 *M*	0.01 *M* §	43	7.31	8.20
8	.....	.....	.....	..	24.11	31.05

* Data of Clowes (1916) except for last two columns, which present data of Harkins and Zollman (1926) on the interfacial tensions of these solutions.

† With 0.001 *N* oleic acid in the oil.

‡ 0.0045 in Harkins' experiments.

§ 0.0060 in Harkins' experiments.

man [60] studied the systems investigated by Clowes in regard to interfacial tension changes, and the last two columns of Table IV show their experimental data. They point out that, when the interfacial tension of a water-benzene system falls below 10 dynes per centimeter, the benzene can be easily emulsified in the aqueous phase, and that, when the interfacial tension falls below 1 dyne per centimeter, emulsions are formed spontaneously. Such emulsions are stable for years.

Roberts (*loc. cit.*) has proposed a theory of ion antagonism in emulsion formation. He suggests that calcium oleate which is soluble in oil will tend to be adsorbed on the oil side and will try to pass its polar end into the water on the opposite interface, whereas sodium oleate in the water will tend to be adsorbed on the water side and will try to pass its non-polar end into the oil. He suggests that this competition for positions across the interface will actually result in a mutually repulsive reaction between the competing molecules so that neither achieves its purpose, provided that they are present in a certain critical ratio. If the ratios differ from the critical ratio, the external phase of the resulting emulsion will depend upon whether or not the calcium soap or the sodium soap is in excess of the critical ratio.

[60] Harkins, W. D., and Zollman, Henrietta, Interfacial Tension and Emulsification. I. The Effects of Bases, Salts, and Acids upon the Interfacial Tension between Aqueous Sodium Oleate Solutions and Benzene. II. Extremely Small Interfacial Tensions Produced by Solutes, *J. Am. Chem. Soc.*, 48: 69–80 (1926).

The antagonistic action of calcium and sodium is well illustrated by the work of Chambers and Reznikoff.[61] Making use of Chambers' micromanipulation apparatus, amoebae were injected with dilute solutions of sodium chloride, potassium chloride, calcium chloride, and magnesium chloride. Injection of sodium chloride or potassium chloride caused a liquefaction of the protoplasm surrounding the injected area. In other words, the protoplasm shows a decreased viscosity, becoming more nearly a sol. On the other hand, when calcium chloride is injected, there is a local solidification of the injected protoplasmic region, producing a more or less rigid mass of gel which the amoeba "pinches off" and rejects. The portion affected is in this way eliminated, leaving the remainder of the amoeba in apparently a normal condition. When magnesium chloride is injected, there is a local solidification similar to that occurring on the injection of calcium chloride. In this case, however, no "pinching off" reaction takes place, and the solidification process gradually spreads throughout the amoeba, causing complete gelation and death. When mixtures of sodium chloride and calcium chloride or when mixtures of potassium chloride and calcium chloride, similar in concentration to the mixtures used by Clowes and by Harkins and Zollman in Table IV, are injected, no effect is observable, the sodium chloride neutralizing the solidifying effect of the divalent salt and the divalent salt neutralizing the liquefying action of the sodium chloride. Chambers and Reznikoff suggest that "at least one of the features of the antagonistic action of NaCl or KCl to $CaCl_2$ is the maintenance in protoplasm of a definite balance between its liquid and solid phases. This phenomenon possibly depends upon the formation of a balanced proportion of Na and Ca or of K and Ca protein salts. It may also be due to the formation of Na or K and Ca soaps." In the light of the experiments of Harkins and Zollman we must conclude that interfacial tension phenomena and alteration in the type of protoplasmic emulsions are features in the experiments noted by Chambers and Reznikoff.

The antagonistic action of ions has been further studied by Chambers and co-workers [62–64] and by Heilbrunn and co-workers.[65–68] Heil-

[61] Chambers, Robert, and Reznikoff, Paul, The Reaction of the Protoplasm of the Living Amoeba to Injected Salts, *Proc. Soc. Exptl. Biol. and Med.*, 22: 320–322 (1925).

[62] Reznikoff, P., and Chambers, R., Micrurgical Studies in Cell Physiology. III. The Action of CO_2 and Some Salts of Na, Ca, and K on the Protoplasm of *Amoeba dubia*, *J. Gen. Physiol.*, 10: 731–738 (1927).

[63] Chambers, R., and Pollack, H., Micrurgical Studies in Cell Physiology. IV. Colorimetric Determination of the Nuclear and Cytoplasmic *p*H in the Starfish Egg., *J. Gen. Physiol.*, 10: 739–755 (1927).

[64] Chambers, R., Kopac, M. J., and Grand, C. G., The Living Cell. Physical Properties of and Microchemical Reactions, *Ind. Eng. Chem.* (Anal. Ed.), 9: 143–145 (1937).

[65] Heilbrunn, L. V., and Daugherty, K., The Action of the Chlorides of

brunn studied changes in viscosity by use of the centrifuge. He found that with the protoplasm of *Amoeba dubia* NaCl alone increased the viscosity of the protoplasm by 41 per cent, KCl alone by 21 per cent, $MgCl_2$ decreased the viscosity by 32 per cent, and $CaCl_2$ decreased the viscosity by 35 per cent. He suggests that the protoplasm micelles are positively charged. With *A. proteus*, Ca^{++} increased viscosity of the plasma gel, whereas K^+, Mg^{++}, and Na^+ decreased viscosity in the order named. When solutions were mixed, antagonism was exhibited. Ca^{++} and K^+ especially neutralized the effects of each other, and he concluded that calcium favors gelation, whereas potassium and magnesium favor solation. He further concludes that magnesium anesthesia is brought about by a liquefaction of the plasma gel and that potassium likewise can induce anesthesia by a similar liquefaction. He finds that the potassium ion is antagonized by hydrogen ions so that at an increased acidity the anesthetic and liquefying action of the potassium ion is not observed.

Weiser [69] studied the antagonistic action of ions in the precipitation of colloid sols, with the interesting result that antagonistic actions similar to the observed biological phenomena were demonstrated. He suggests that possibly the antagonistic action of salt pairs (*e.g.* Na *vs.* Ca) may be conditioned on the effect of the ions on the permeability of the colloidal film making up the cell membrane.

Bull and Gortner [70] investigated the *zeta*-potential as a factor in ion antagonism in salt pairs and could not observe any antagonistic effects. However, when the data were recalculated [71] in terms of absolute charge on the surface, a marked ion antagonism was found between NaCl : KCl, KCl : $MgCl_2$, NaCl : $MgCl_2$, NaCl : $CaCl_2$, KCl : $CaCl_2$, and $CaCl_2$: $MgCl_2$, so that perhaps the ratio of the ions contributing to the absolute charge at the interface may be at least one of the determining factors in the ion antagonisms which have been observed in biological systems. The last word has not been written on the question of ion antagonism as exhibited in biological systems,

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Sodium, Potassium, Calcium, and Magnesium Ions on the Protoplasm of *Amoeba dubia, Physiol. Zool.*, 4: 635–651 (1931).

[66] Heilbrunn, L. V., and Daugherty, K., The Action of Sodium, Potassium, Calcium, and Magnesium Ions on the Plasmagel of *Amoeba proteus, Physiol. Zool.*, 5: 254–274 (1932).

[67] Heilbrunn, L. V., Magnesium and Potassium Anaesthesia in Amoeba, *Proc. Soc. Exptl. Biol. Med.*, 29: 467–469 (1932).

[68] Heilbrunn, L. V., and Daugherty, K., A Further Study of the Action of Potassium on Amoeba Protoplasm, *J. Cell. Comp. Physiol.*, 5: 207–218 (1934).

[69] Weiser, Harry B., The Antagonistic Actions of Ions in the Neutralization of Sols. II, *J. Phys. Chem.*, 30: 1527–1537 (1926).

[70] Bull, H. B., and Gortner, R. A., Studies on Electrokinetic Potentials. VIII. Ion Antagonism, *J. Phys. Chem.*, 35: 700–721 (1931).

[71] Moyer, L. S., and Bull, H. B., Electrokinetics. XVII. Surface Charge and Ion Antagonism, *J. Gen. Physiol.*, 19: 239–247 (1935).

but the solution of the phenomenon will undoubtedly be hastened when the methods of colloid chemistry are applied to such systems.

The breaking of emulsions and the inversion of emulsions involve changes in the interfacial tension between the two components and an alteration in the orientation of the surface film surrounding the suspended droplets. It sometimes happens that crude petroleum issues from the ground emulsified with the salt water associated with the petroleum fields. In such instances the emulsifying agent appears to be asphaltic residues. Obviously such emulsions are without commercial value, and the breaking of such emulsions becomes an important industrial problem. A knowledge of surface chemistry and of the role that interfacial tension plays in emulsion formation, as well as a knowledge of the antagonistic effects of ions and of polar and nonpolar groups in organic compounds, has proved very helpful to the chemist who is faced with such industrial problems. Specific instances will be cited under molecular orientation (*vide infra*).

As noted earlier, only a dilute emulsion results when we have oil dispersed in pure water. This is due to the fact that we have present in such a system a relatively high interfacial tension. Such systems behave as true lyophobic sols. When the interfacial tension is lowered by the introduction of an emulsifying agent, an extremely high concentration of the disperse phase can be obtained. Such emulsions behave more or less as lyophilic systems and take on to a large degree the properties characteristic of the emulsifying agent. It is such systems that are usually called emulsions, and the fact that the stabilizing agent is generally a lyophilic colloid justifies our including emulsions in a consideration of colloid systems.

CHAPTER III

CERTAIN PHYSICAL PROPERTIES CHARACTERISTIC OF COLLOID SYSTEMS

VISCOSITY AND PLASTICITY.—Viscosity may be defined as the internal friction of a liquid, the resistance to shear or flow. The unit of viscosity is the *poise,* so named after the Frenchman, Poiseuille,[1] who first devised methods for the measurement of viscosity. *A poise may be defined in c.g.s. units as the force (one dyne) which, when exerted on a unit area between two parallel planes one square centimeter in area and one centimeter apart, produces a difference in streaming between the two planes of one centimeter velocity per second.* Inasmuch as the absolute viscosity of water at 20° C. is 1.0050 centipoise, the centipoise is generally used as the unit for plotting the viscosity of liquid systems. Viscosity is expressed in absolute terms and is not derived from a reference liquid. Too often there is a general misconception that water is the reference liquid to which viscosity units are referred. Bingham[2] has recorded the viscosity of various solutions which can be used as standard solutions for the calibration of an uncalibrated viscosimeter. Various types of viscosimeters are available.

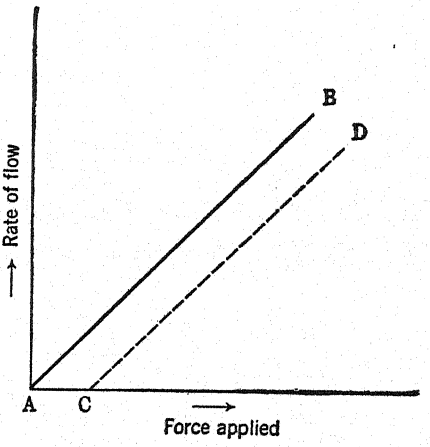


FIG. 9.—Showing diagrammatically the relation between true viscosity and plasticity. *AC* is the "yield value" of the plasticity formula.

The solid line *AB* in Fig. 9 is a diagrammatic representation of a truly viscous system. The increase in rate of flow with increase in

[1] Poiseuille, Recherches expérimentales. Sur le mouvement des liquides de nature différente dans les tubes de très-petits diamètres. *Ann. chim. phys.*, 3 ser., 21: 76–110 (1847).

[2] Bingham, E. C., and Jackson, R. F., Standard Substances for the Calibration of Viscosimeters, U. S. Bur. Standards, Scientific Paper 298 (1917).

force applied is a linear function which passes through the point of origin. The dotted line *CD* represents plastic flow and intersects the force axis to the right of zero force. It is accordingly necessary that energy be added to deform the system before plastic flow begins. This amount of energy is known as the *yield value.* Plasticity, therefore, differs from viscosity in that when we are dealing with plastic materials we must determine not only the rate of flow per unit of force applied, but likewise the yield value. This phase has been considered at length by Bingham and his co-workers.[3, 4, 5]

Colloid systems show a wide range in viscosity or plasticity. In general, lyophobic sols exhibit a viscosity which approaches very closely to the viscosity of the pure dispersions medium, and which increases only slightly with increasing concentration of dispersed material. On the other hand, lyophilic systems may reach very high values for viscosity, and many lyophilic sols and gels are truly plastic and have relatively high yield values. With lyophilic systems we do not have a linear relationship between viscosity or plasticity and the concentration of a disperse phase but rather a parabola, such as is shown diagrammatically in Fig. 10. This is due to the fact that the lyophilic systems are solvated, and accordingly the particle has associated with it a larger or smaller amount of the dispersions medium, so that the actual amount of the dispersions medium is decreasing with each added increment of disperse phase. Sharp and Gortner [6] have summarized certain of the

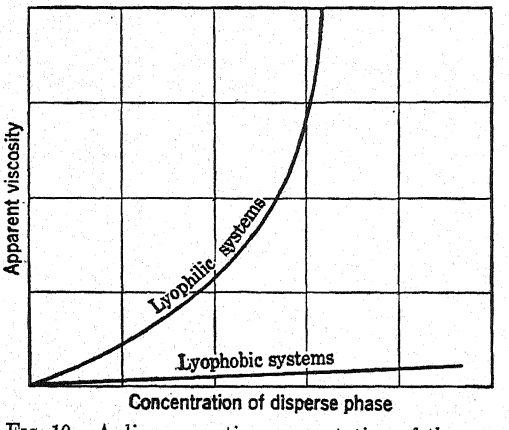


FIG. 10.—A diagrammatic representation of the relation between viscosity and concentration in lyophobic and lyophilic systems.

[3] Bingham, E. C., An Investigation of the Laws of Plastic Flow, U. S. Bur. Standards, Sci. Paper No. 278 (1916).

[4] Bingham, E. C., Fluidity and Plasticity, McGraw-Hill Book Company, New York (1922).

[5] Bingham, E. C., and Durham, T. C., The Viscosity and Fluidity of Suspensions of Finely-Divided Solids in Liquids, *Am. Chem. J.*, 46: 278–297 (1911).

[6] Sharp, P. F., and Gortner, R. A., Viscosity as a Measure of Hydration Capacity of Wheat Flour and its Relation to Baking Strength, Minnesota Agricultural Experiment Station Technical Bulletin No. 19 (1922).

literature dealing with the viscosity of colloidal systems. Excellent discussions have also been presented by Ostwald,[7, 8] and by Hatschek.[9-13] Bingham [14] has likewise given a general review.

As a general rule the viscosity of colloid systems decreases as temperature increases. This is due in part to the effect of temperature on the dispersions medium. Water has a viscosity of 0.2838 centipoise at 100° C., whereas at 0° C. it has a viscosity of 1.7921 centipoise. The viscosity has increased approximately 800 per cent from 100° to 0°. Accordingly one reason why a precipitate can be washed more readily with boiling water than with cold water lies in the fact that hot water passes more readily through the pores of the filter paper, owing to the lowered viscosity of the water. The question as to whether a precipitate should be washed with hot water or with cold water depends upon two factors, the change in solubility of the material with temperature and the change of viscosity of water with temperature. If the change in the viscosity of the water is greater than the change in solubility, more efficient and more rapid washing can be effected with a given volume of water by using hot water instead of cold water. On the other hand, if the change in temperature increases the solubility of the precipitate more rapidly than the viscosity changes, hot water should be avoided in order to obtain the maximum yield of the desired precipitate.

With colloid systems, viscosity changes due to temperature are influenced not only by the viscosity of the dispersions medium but likewise by the effect of temperature on solvation. Most lyophilic colloids are more highly solvated at the lower temperatures. Gelatin and agar form relatively non-viscous sols at the higher temperatures but set to more or less rigid or plastic gels at the lower temperatures. Starch, on the other hand, forms a more or less lyophobic suspension at

[7] Ostwald, Wo., The Importance of Viscosity for the Study of the Colloidal State, *Trans. Faraday Soc.* 9: 34–46 (1913).

[8] Ostwald, Wo., Über die Bedeutung der Viskosität für das Studium des kolloiden Zustandes, *Kolloid Z.*, 12: 213–222 (1913).

[9] Hatschek, E., Die Viskosität der Dispersoide, *Kolloid Z.*, 7: 301–304 (1910).

[10] Hatschek, E., Die allgemeine Theorie der Viskosität zweiphasiger Systeme, *Kolloid Z.*, 12: 238–246 (1913).

[11] Hatschek, E., The General Theory of Viscosity of Two Phase Systems, *Trans. Faraday Soc.*, 9: 80–92 (1913).

[12] Hatschek, E., The Viscosity and Hydration of Colloidal Solutions, *Biochem. J.*, 10: 325–330 (1916).

[13] Hatschek, E., The Viscosity of Colloidal Solutions, Chap. XLVI of Colloid Chemistry, Theoretical and Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, New York (1926).

[14] Bingham, E. C., Fluidity and Plasticity, Chap. XLIV of Colloid Chemistry, Theoretical and Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, New York (1926).

the lower temperatures, and the temperature may be increased appreciably without great changes in viscosity until a critical temperature, the gelatinization point, is reached. At this critical point the starch granules undergo rapid hydration and the extremely viscous or plastic starch paste results. An increase in temperature past this point results in a decreased viscosity or plasticity, due almost wholly to a decrease in the viscosity of the dispersions medium, since the starch micelles in a starch paste at 25° have the same volume as at 90°.[15]

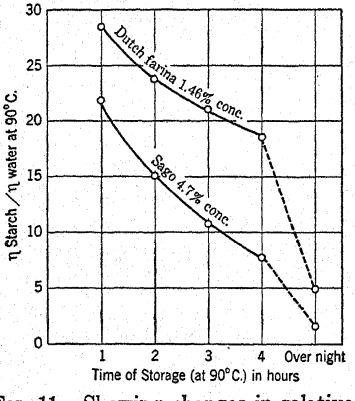


FIG. 11.—Showing changes in relative viscosity of gelatinized starch systems with time. (Data of Farrow and Lowe.)

Time may have a great effect on plasticity or viscosity of colloid systems. Farrow and Lowe[16] investigated the changes in viscosity of a starch paste with time. Figure 11 shows a typical curve taken from their data. This change in viscosity is undoubtedly associated with a decrease in the solvation of the micelles.

Electrolytes may greatly alter the viscosity of lyophilic systems, in some instances causing relatively enormous changes. In order for such results to be manifest, it is essential that colloid systems be as nearly as possible electrolyte-free. Figure 12[17] shows the enormous decrease in the viscosity of an acidulated wheat flour-water system on the addition of a trace of the salt of a bivalent metal. Similar effects are common when biochemical systems are under investigation.

The viscosity of a crystalloidal solution of a given solute in a given solvent is determined solely by the concentration and the temperature at which the measurement is made. This is not true for lyophilic colloid systems. Ostwald[18, 19] has pointed out that in lyophilic systems there are at least ten factors which must be taken into considera-

[15] Gortner, R. A., The Hydration Capacity of Starch, *Cereal Chem.*, 10: 298–312 (1933).

[16] Farrow, F. D., and Lowe, G. M., The Flow of Starch Paste through Capillary Tubes, *J. Textile Institute*, 14: 414–440 (1923).

[17] Sharp, P. F., and Gortner, R. A., The Physico-chemical Properties of Strong and Weak Flours. V. The Identity of the Gluten Protein Responsible for the Changes in Hydration Capacity Produced by Acids, *J. Phys. Chem.*, 27: 674–684 (1923).

[18] Ostwald, Wo., The Importance of Viscosity for the Study of the Colloidal State, *Trans. Faraday Soc.*, 9: 34–46 (1913).

[19] Ostwald, Wo., Über die Bedeutung der Viskosität für das Studium des kolloiden Zustandes, *Kolloid Z.*, 12: 213–222 (1913).

tion: (1) concentration, (2) temperature, (3) degree of dispersion, (4) solvation, (5) electrical charge, (6) previous thermal treatment, (7) previous mechanical treatment, (8) the presence or absence of traces of other lyophilic colloids, (9) the age of the lyophilic sol, and (10) the presence of both electrolytes and non-electrolytes. Another factor of extreme importance should be added to this list, *i.e.*, the rate of shear. It is obvious from the above list of variables that the study of viscosity or plasticity in lyophilic systems presents experimental difficulties. On the other hand, viscosity and plasticity methods afford one of the most valuable tools available to the colloid chemist.

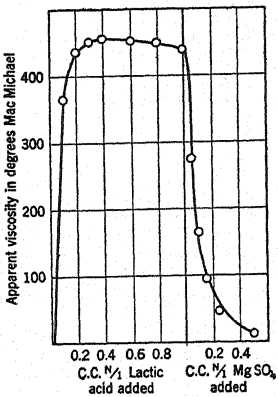


FIG. 12. — Showing the changes in apparent viscosity produced in a flour-water suspension by acidulation followed by the addition of an inorganic salt solution. (Data of Sharp and Gortner.)

In contrast to the linear relationships of truly viscous flow, the colloid chemist is continually discovering unexpected changes in the apparent viscosity of the systems with which he deals. Figure 13 [20] illustrates the peculiar form of the viscosity curve yielded by egg white during the process of heat coagulation. The reasons for the very peculiar curve are still unexplained. Figure 14, taken from the same paper by Wo. Ostwald, shows the viscosity curve for the gelatinization of starch.

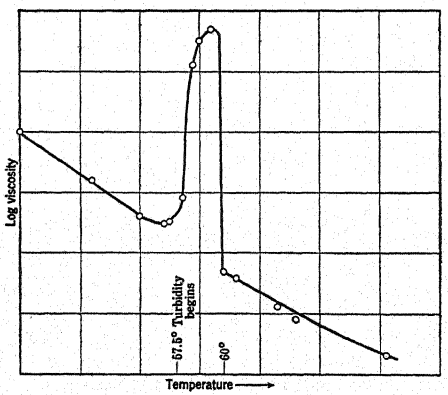


FIG. 13.—Showing viscosity changes which accompany the heat-coagulation of egg albumin. (Data of Wo. Ostwald.)

[20] Ostwald, Wo., *Kolloid Z.*, 12: 217 (1913).

Poiseuille first developed from theoretical considerations an equation for viscosity based on the flow of a liquid through a capillary tube, on the theory that such a column of liquid was composed of innumerable cylinders, each moving slightly faster than the other as the distance from the wall of the tube increased. Stokes,[21, 22] and Hagenbach [23] modified the original equation of Poiseuille to the equation now generally accepted as representing truly viscous flow:

$$\eta = \frac{\pi r^4 pt}{8lV} \tag{4}$$

where η = the coefficient of viscosity;
r = the radius of the tube;
p = hydrostatic pressure of the liquid flowing through the tube;
t = the time necessary for the volume (V) to flow through the capillary;
l = length of the capillary.

In unit time (1 second) this equation becomes):

$$V = \frac{\pi r^4 p}{8l\eta} \tag{5}$$

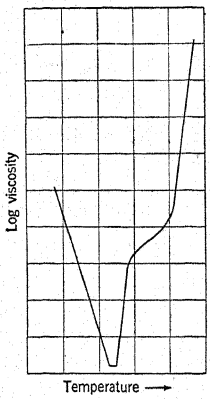


FIG. 14. — Showing viscosity changes which accompany the heat-gelatinizing of starch. (Data of Wo. Ostwald.)

This equation is very satisfactory for pure liquids and for truly crystalloidal solutions. Even for these, however, deviations from the equation may be observed at high pressures, when the flow is more like a solid rod of liquid being forced through without the accompanying internal friction, a film of liquid practically without motion acting as a lubricant on the walls of the tube.

The material comprising the walls of the capillary tube makes no appreciable difference in the viscosity of truly viscous systems. Apparently there is always a thin film of liquid on the surface of the capillary tube, so that friction between the liquid and the wall of the capillary does not exist. The friction is actually between the bulk of the liquid flowing through the tube and a very thin film of liquid fixed on the surface of the capillary.

[21] Stokes, G. G., On Some Cases of Fluid Motion, *Trans. Camb. Phil. Soc.*, 8: Pt. I, 105–137 (1844).

[22] Stokes, G. G., On the Theories of the Internal Friction of Fluids in Motion and of the Equilibrium and Motion of Elastic Solids, *Trans. Camb. Phil. Soc.*, 8: Pt. III, 287–319 (1847).

[23] Hagenbach, E., Über die Bestimmung der Zähigkeit einer Flüssigkeit durch den Ausfluss aus Röhren, *Pogg. Ann. Phys. Chem.*, 109: 385–426 (1860).

The above discussion is necessarily abbreviated and does not include a consideration of many of the factors which must be taken into account in making measurements of absolute viscosity. Barr [24] has considered all these factors in detail in his monograph and points out that in the design of a capillary viscosimeter it is important that there should be a certain ratio between the bore and the length of the capillary tube, thus bringing the time of outflow into the range where the kinetic energy correction becomes small.[25]

Torsion viscosimeters [26, 27] show particular adaptibility to rapid determinations of the viscosity of industrial materials. The principle of the torsion viscosimeter is to rotate the liquid in question in a cup at a constant rate of speed, at the same time suspending a cylinder in the liquid by means of a torsion wire. The friction of the rotating liquid on the cylinder immersed in it twists the torsion wire a certain number of degrees. The torc of the wire measured in angular degrees can be related to the viscosity of the liquid.

More recently the falling-ball viscosimeters have been perfected. These depend upon the rate of fall of a sphere through a tube of the liquid under investigation, together with the appropriate application of Stokes' law to the measurements obtained. The advantage of the falling-ball viscosimeters is that the specific gravity of the ball can be varied through a wide range depending upon the nature of the material from which the ball is constructed, and accordingly such viscosimeters can be used to measure viscosity over the range of 0.65 to 100,000 centipoises with a precision of $\pm$ 0.75 per cent. The monograph by Barr may be consulted for the theory of the measurement and the literature of the method.

No adequate equation has been devised which expresses the absolute viscosity of a lyophilic system. Einstein [28] proposed the equation:

$$\eta' = \eta_0(1 + K\phi) \qquad (6$$

[24] Barr, G., A Monograph of Viscometry, Oxford University Press, London (1931).

[25] The effect of obstructions, constrictions, or irregularities in the tube on the planes of streaming within the liquid flowing through the tube are beautifully illustrated in the photographs reproduced in a series of papers by Dupin *et al.* (*J. Rheol.*, 3: 415–436, 1932). In certain of the experiments aluminum powder was placed in the liquid and the planes of flow photographed, thus visualizing the actual paths of flow.

[26] Hatschek, E., Die allgemeine Theorie der Viskosität zweiphasiger Systeme, *Kolloid Z.*, 12: 238–246 (1913).

[27] Herschel, W. H., The Macmichael Torsional Viscosimeter, *J. Ind. Eng. Chem.*, 12: 282–286 (1920).

[28] Einstein, A., Eine neue Bestimmung der Moleküldimensionen. *Ann. Physik*, 4 Folge, 19: 289–306 (1906).

where η' = the viscosity of the lyophilic sol;
η_0 = the viscosity of the dispersions medium;
ϕ = the percentage of the system occupied by the volume of the disperse phase;
K = a constant.

In his original paper Einstein suggested that K was approximately unity. Later he [29] suggested that the value of K was more nearly 2.5. Hatschek [30] first suggested a constant of 4.5 for the Einstein equation and later [31] proposed a somewhat different equation:

$$\frac{\eta}{\eta_0} = \frac{1}{1 - \sqrt[3]{\phi}} \tag{7}$$

Kunitz [32] suggested a slightly different equation:

$$\frac{\eta}{\eta_0} = \frac{1 + 0.5\phi}{(1 - \phi)^4} \tag{8}$$

and has applied it to a variety of systems. For glycogen sols varying in concentration from 20 to 40 per cent, Kunitz found the specific volume (ϕ/C) to vary from 1.16 to 1.25, whereas, when the Hatschek equation was applied, the variation was from 1.86 to 2.21. Sucrose in concentrations from 1.00 to 21.7 per cent varied in specific volume only from 0.60 to 0.63, when determined by the Kunitz equation, whereas it varied from 1.04 to 1.69 when the Einstein equation was applied. Casein sols in the concentration range from 4.35 to 9.39 per cent showed a constant specific volume of 5.5 by the Kunitz equation and a specific volume range from 8.04 to 9.72 by the Hatschek equation.

Kunitz, Anson, and Northrop [33] studied the hydration, molecular weight, and molecular volume of a number of proteins in solution. Osmotic pressure was determined by direct measurements, thus securing particle weight. The size of the particle was determined by measuring the rate of diffusion, and from this the density of the particle in solution was calculated. The difference between this value and the dry value enabled them to calculate the degree of hydration. The degree of hydration was also determined by viscosity measurements,

[29] Einstein, A., Berichtigung zu meiner Arbeit "Eine neue Bestimmung der Moleküldimensionen," *Ann. Physik*, 4 Folge, 34: 591–592 (1911).

[30] Hatschek, E., Viskosität der Dispersoide, *Kolloid Z.*, 7: 301–304 (1910).

[31] Hatschek, E., Die Viscosität von Blutkörperchen-Suspensionen. *Kolloid Z.*, 27: 163–165 (1920).

[32] Kunitz, M., An Empirical Formula for the Relation between Viscosity of Solution and Volume of Solute, *J. Gen. Physiol.*, 9: 715–725 (1926).

[33] Kunitz, M., Anson, M. L., and Northrop, J. H., Molecular Weight, Molecular Volume, and Hydration of Proteins in Solution, *J. Gen. Physiol.*, 17: 365–373 (1934).

applying the Kunitz equation. Hemoglobin had an apparent particle weight of 67,000, a particle radius of 2.73 mμ and an apparent hydration ranging from zero to 0.14 gram water per gram protein. Hydration from viscosity data was 0.13 gram water per gram protein. Isoelectric gelatin had a particle weight of 61,500, a particle radius of 5.4 mμ, and an apparent hydration of 5.8 grams water per gram protein. From viscosity data the hydration was 5.9 grams water per gram protein. Crystalline trypsin had a particle weight of 35,000, a particle radius of 2.60 mμ, and an apparent hydration of 0.54 gram water per gram protein. Viscosity data indicated 0.49 gram water per gram protein.

Gortner [34] applied the Kunitz equation to the study of the hydration capacity of starch. Since the formula is a fourth-degree equation, it is necessary to plot the theoretical curve on cross-section paper and use this theoretical graph for the determination of the percentage of the sol which is occupied by the disperse phase at the values of viscosity which are experimentally determined. Table V gives the essential data from which the theoretical curve can be plotted.

TABLE V

VALUE OF RELATIVE VISCOSITY (η/η_0) AND VOLUME OF SOL. (ϕ) OCCUPIED BY THE DISPERSE PHASE FOR PLOTTING THE CURVE OF THE EQUATION $\eta_r = \frac{1 + 0.5\ \phi}{(1 - \phi)^4}$

ϕ	η_r	ϕ	η_r
0	1.000	60	50.781
10	1.600	62	62.830
20	2.686	64	78.589
30	4.790	66	99.526
40	9.274	68	127.807
42	10.692	70	166.677
44	12.405	72	221.30
48	16.959	74	299.80
50	20.000	76	415.94
52	23.736	78	593.37
54	28.364	80	875.00
56	34.151	..	.....

When the Kunitz equation was applied to Samec's [35] viscosity data of starches of different botanical origins, the data in Table VI were obtained. Why starches from different botanical origins show such a

[34] Gortner, R. A., The Hydration Capacity of Starch, *Cereal Chem.*, 10: 298–312 (1933).

[35] Samec, M., Kolloidchemie der Stärke, Theo. Steinkopff, Dresden and Leipzig (1927).

wide range of hydration capacities is a problem which awaits elucidation.

TABLE VI

THE HYDRATION AT 25° C. OF STARCHES FROM VARIOUS BOTANICAL SOURCES

(All samples gelatinized in 2 per cent concentration for 1 hour at 120° C. viscosities on 1 per cent sols at 25° C. Calculation from data of Samec and Haerdtl)

	η_r	ϕ	Volume Occupied by 1 Gram
		Per cent	*Cc.*
Potato (fat extracted, Kahlbaum)	6.63	35.00	35.00
Potato (ordinary)	5.05	30.75	30.75
Meadow-saffron (*Colchicum autumnale*)	3.34	28.33	28.33
Arrowroot (*Maranta arundinacea*)	3.13	22.75	22.75
Cassava (*Manihot utilissima*)	2.72	20.20	20.20
Zedoary (*Curcuma Zedoaria*)	2.59	19.40	19.40
Horse-chestnut (*Aesculus hippocastanum*)	2.47	18.50	18.50
Wheat (*Triticum vulgare*)	2.10	15.50	15.50
Glutinous rice (*Oryza glutinosa*)	1.96	14.50	14.50
Maize (*Zea mays*)	1.60	10.25	10.25
Rice	1.48	8.5	8.5

The Kunitz equation with reference to the problem of starch gelatinization has been further studied by Clark,[36] who comes to the conclusion that the equation is purely empirical inasmuch as, when corrections are made for the kinetic energy of the liquid flowing through the tube, the volume ϕ varies widely over a concentration range, whereas it is essentially a constant when the kinetic energy correction is not applied. There is, however, a considerable body of data which indicates that the Kunitz equation is the best approximation which has yet been devised for studying the viscosity of lyophilic systems and particularly for investigating starch phenomena.

When we pass to plastic systems we must take into consideration the yield value. As already noted, the yield value indicates the pressure which must be exerted before the theoretical flow will start. The term *consistency*, or its reciprocal, *mobility*, is used in place of viscosity to indicate that we are dealing with plastic flow. In its simplest terms the equation for viscosity may be expressed as,

$$\frac{V}{P} = K \tag{9}$$

[36] Clark, Ronald E., Physico-Chemical Studies on Starch Gelatinization, Ph.D. Thesis, University of Minnesota, June, 1937.

where V = volume of liquid passing through the capillary per second;
P = pressure in grams per square centimeter producing the flow;
K = a constant.

This, in terms of plasticity, becomes

$$\frac{V}{P - p} = K_1 \tag{10}$$

where p = yield value pressure in grams per square centimeter.

Bingham and White [37] give a somewhat more elaborate equation for viscous flow.

$$\eta = \frac{\pi G r^4 P t}{8V(l + \lambda)} - \frac{MDV}{8\pi t(l + \lambda)} \tag{11}$$

where η = coefficient of viscosity;
G = gravitational constant;
P = pressure in grams per square centimeter;
t = time in seconds;
V = volume of flow;
r = radius of capillary in centimeters;
l = length of capillary in centimeters;
D = density of the liquid;
M = a constant whose value was taken as 1.12 by Bingham and White;
λ = a correction to be added to the length of the capillary to correct for the viscous flow outside of the capillary.

The expression $\frac{MDV}{8\pi t(l + \lambda)}$ is subtracted to correct for the kinetic energy of the liquid emerging from the capillary. This correction is usually extremely small, approaching zero, and may be disregarded in a consideration of plastic flow. Neither is it necessary to correct for the resistance to flow outside the capillary, so that the equation for plastic flow may be written

$$\text{Consistency} = \frac{\pi r^4 G(P - p)t}{8lV} \tag{12}$$

Plasticity is usually expressed in terms of mobility (μ) which is the reciprocal of the consistency. Kelly [38] derived a somewhat different equation which he applied to the study of plasticity of rubber sols,

[37] Bingham, E. C., and White, G. F., Fluidität und die Hydrattheorie. I. Die Viskosität von Wasser, *Z. physik. Chem.*, 80: 670–686 (1912).

[38] Kelly, W. J., The Plasticity of Rubber and its Sols. I. Colloid Symposium Monograph, Vol. III, pp. 303–316 (1925).

and suggests that by altering the method of measurement and using a differential equation, fluidity values accurate to within 1 per cent can be obtained. Kelly's original paper should be consulted for the details of the method. His equation for fluidity is

$$\phi_2 = \frac{\frac{\Delta V}{\Delta t}}{\frac{\pi P r^4}{\frac{8(l_2 + l_1)}{2}}} \qquad (13)$$

where ϕ_2 = the fluidity at a point midway between two calibrations, l_1 and l_2, on the capillary tube, the other terms in the equation being identical with the terms used in the equation above.

Sharp [39] applied plasticity methods to a study of flour-in-water suspensions similar to those studied earlier by Sharp and Gortner, and found that even simple flour-in-water suspensions are plastic when they contain 9 per cent or more of flour by weight on the dry basis. The introduction to Sharp's paper summarizes some of the more important literature dealing with plastic flow. The later papers by Karrer [40] may also be consulted.

Recently a new type of plasticity technic has been applied to industrial problems. Bailey [41] attached a microwatt-hour meter to a small dough-mixing machine and measured the electrical power necessary to turn the blades of the mixing machine for 100 revolutions in a bread dough. Dieterich [42] has used a somewhat similar instrument to measure the plasticity changes which occur in rubber on the milling rolls, and the Brabender farinograph employs a somewhat similar principle and has proved of great value in the cereal industry. In the farinograph the motor is mounted free-floating, and the thrust of the blades in the dough-mixing chamber rotates the motor. An arm attached to the motor frame draws on a kymograph a continuous record of the thrust which the blades encounter, thus affording a record of the consistency of the dough throughout the entire mixing process. Fig-

[39] Sharp, P. F., Wheat and Flour Studies V. Plasticity of Simple Flour-in-water Suspensions, *Cereal Chem.*, 3: 40–56 (1926).

[40] Karrer, E., A New Plastometer, *Ind. Eng. Chem.* (*Anal. Ed.*), 1: 158–160 (1929); Meaning and Measurement of Plasticity, *Ind. Eng. Chem.*, 21: 770–773 (1929).

[41] Bailey, C. H., A Method for Measuring the Relative Plasticity of Pastes and Doughs, *J. Rheol.*, 1: 429–432 (1930).

[42] Dieterich, E. O., Time and Temperature-plasticity Relations for Crude Rubber as Measured with the Goodrich Plastometer, *Ind. Eng. Chem.*, 21: 768–770 (1929).

ure 15 shows such a record for three representative bread doughs made from different flours. Flour A is characteristic of a flour which "develops" its gluten after a few minutes of mixing and with continued mixing maintains a high consistency for a long period of time. Flour B develops initially a somewhat higher consistency than Flour A, but the dough breaks down more rapidly with continued mixing. Flour C suddenly develops a high consistency, followed by an equally sudden and drastic breakdown of the gel structure. Flour A is typical of the high-grade northern spring wheats and is a good bread flour. Flour C is extremely sensitive to the mixing process and is a typical pastry flour. Flour B is intermediate in its characteristics between Flours A and C.

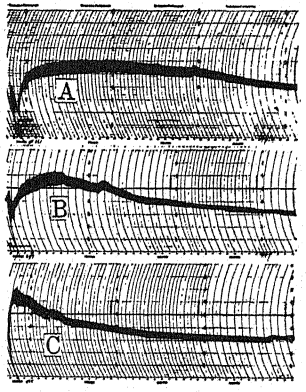


FIG. 15.—Brabender farinograph plasticity-time curves of three wheat-flour doughs prepared from flours having different colloidal properties.

HYSTERESIS. — Hysteresis, in colloid terminology, is not synonymous with the same term in engineering practice. Hysteresis is used by the colloid chemist in expressing in a single term the influence of the previous history of a colloid system on its present behavior. It has been allegorically referred to as the "memory" of a colloid system. In many instances the past history of a colloid system very largely determines its present behavior. This is especially true of lyophilic gels.

Gortner and Hoffman[43] studied the effect of the rehydration of gelatin sheets and gelatin granules which had been dried down to approximately 3 per cent moisture content from gels of different initial concentrations. Figure 16 shows the *grams water imbibed per gram dry gelatin* for uniform-sized gelatin particles (retained on a 1-mm. sieve but passing a 2-mm. sieve) which had been dried down from 10, 25, and 35 per cent gelatin gels. The rate of imbibition for the original commercial gelatin received from the manufacturer is also indicated in the figure.

Later they (*loc. cit.*) studied the hysteresis effects in dried gelatin, part of which had been dried from gelatin sols at 45° C., the remainder having been dried at a temperature below the gelating point, so that it had remained in the gel form throughout the entire period of drying.

[43] Gortner, R. A., and Hoffman, W. F., Evidence of a Structure in Gelatin Gels, *Proc. Soc. Exptl. Biol. and Med.*, 19: 257–264 (1922); and The Imbibition of Gelatin Dried as a Gel and as a Sol, *J. Phys. Chem.*, 31: 464–466 (1927).

Table VII shows the results obtained. These experiments show that the previous treatment has a profound effect on the rate of hydration of gelatin particles. Similarly, if gelatin gels are "melted" and allowed to resolidify, and this process is repeated a number of times, it will be found that the "melting-point" temperature becomes progressively lower and the "solidifying-point" temperature is likewise lowered. In a similar manner, rubber can be stretched and released a number of times so that it eventually changes its shape and becomes permanently distorted. Starch pastes on standing undergo retrogression with a loss in viscosity or plasticity and a great loss in water-imbibing capacity (cf. Fig. 11).

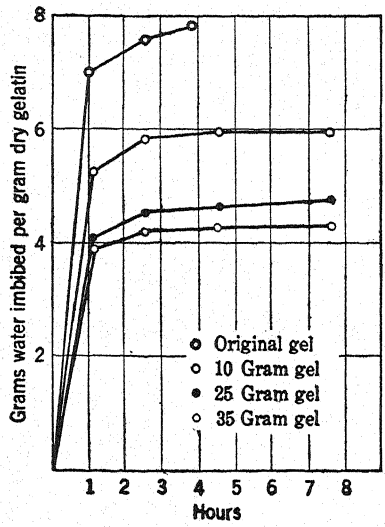


FIG. 16.—Imbibition curves of uniform-sized granules of gelatin which had been dried down to approximately 3 per cent moisture content from gels of different initial concentrations and then again allowed to swell in water. (Data of Gortner and Hoffman.)

Temperature relations sometimes play an unexpected role in determining the properties of the resulting gel.[44] Thus, a gelatin gel allowed to set at 0.0° C. and kept at 0.0–1.0° C. for 48 hours was transformed into a sol in 13 minutes when the temperature was raised to 22.5° C., whereas an identical gelatin sol allowed to set at 14.5–15.0° C. and kept at that temperature for 48 hours "melted" at 22.5° C. only after having been maintained at that temperature for 231 minutes.

That hysteresis effects influence the behavior of natural biological gels is evidenced by the observations of Bailey and Bayfield[45] that wheat berries, which have been frosted when in a moist, immature stage or when they have a fairly high water content, have, after harvesting and drying, a much higher hydration capacity and that such frosted wheat tends to imbibe water more readily and to reach a much higher water content at equilibrium than unfrozen wheat harvested in a normally dry condition. Furthermore, the amount of water taken up by normal wheat after harvesting and drying closely reflects the moisture content of the wheat at the time of harvest, provided that at

[44] Olsen, A. G., Evidence of Structure in Gelatin Gels, *J. Phys. Chem.*, 36: 529–533 (1932).

[45] Bailey, C. H., and Bayfield, E. G., Water Imbibition of Frosted Wheat, *Cereal Chem.*, 7: 108–116 (1930).

TABLE VII

THE IMBIBITION OF UNIFORM SIZED PARTICLES OF GELATIN DRIED AS A "GEL" AND AS A "SOL"

Source of Gelatin Particles, Per Cent	Grams Water Imbibed per Gram Dry Gelatin *							
	1 Hr.	2 Hr.	3 Hr.	4 Hr.	6 Hr.	8 Hr.	12 Hr.	24 Hr.
Gelatin Sol								
5	9.12	10.98	11.97	12.60	12.94	13.69	15.32	16.50
10	3.69	5.65	6.63	7.35	8.23	9.30	10.78	12.13
15	3.64	5.02	5.72	6.45	7.54	8.95	10.76	12.18
20	3.64	4.89	5.73	6.44	7.60	8.70	10.10	11.32
Gelatin Gel								
5	7.27	9.72	10.97	11.75	12.81	14.16	17.17	18.46
10	5.33	7.03	8.21	9.00	10.28	11.38	13.71	14.63
15	5.32	6.70	7.59	8.48	9.12	10.52	12.10	13.35
20	4.19	5.88	6.84	7.45	8.50	9.88	12.06	12.96

* Average of duplicate determinations. In this experiment the gelatin was ground to pass a 2-mm. mesh and was all retained on a 1-mm. mesh.

the time of harvest the wheat contained 40 per cent or more of moisture. In other words, "the water-imbibing capacity of entire wheat kernels was found to be related to their previous history," and "the level of water content tends to return to that of the grain at the time it was harvested." Bailey and Bayfield propose the imbibition capacity of wheat as an approximate measure of the extent to which the kernels have been damaged by premature freezing.

The phenomenon of hysteresis undoubtedly plays a very great role in biological organisms, and when the individual factors are ultimately analyzed, it will certainly be found that the behavior of the lyophilic colloids which are present is predominantly responsible for the reactions which are observed. Hysteresis effects are most striking when one employs viscosity or plasticity methods. Accordingly, in using such methods, the past history of the colloidal system under investigation may well be the most important variable, and it is an essential precaution to duplicate, so far as possible, every detail of mechanical, thermal, or electrical treatment, as well as to observe exact time intervals, if one is to obtain reliable data regarding the viscosity or plasticity of lyophilic colloid systems. Apparently the most striking effects of hysteresis are noted in studies of degree of solvation.

BROWNIAN MOVEMENT.—In 1827, a botanist, Robert Brown, noted that pollen grains suspended in a liquid on the microscope stage were in continuous motion in the liquid. In order to ascertain whether or not such motion was characteristic of living pollen grains, he secured,

from the herbarium, pollen and plant spores which were more than one hundred years old and found that the rate of motion was in no way diminished. It was later observed that any particle of small enough size to remain in more or less permanent suspension in a liquid would exhibit this characteristic motion which has been called the Brownian movement. Particles up to 4 μ and accordingly easily visible in the microscope show the characteristic Brownian motion, but the motion is not so active or so rapid as in colloidal systems.

Burton [46] has given an excellent presentation of the historical phases and theoretical significance of Brownian movement.

A particle in a Brownian movement oscillates rapidly in a haphazard manner around a mean position so that the velocity of movement must be calculated from observations occupying very short periods of time. This has been done by Svedberg, who employed a motion-picture camera to record changes in the position of a particle. Measurements of displacement on the film indicate that particles 40–50 mμ in diameter in a platinum hydrosol may reach a velocity as great as 320 μ per second. This velocity in relation to the size of the platinum particle approximates the velocity of a low-speed rifle ball.

Various theories were devised in order to account for Brownian movement. These theories have been adequately discussed by Burton. It is now definitely known that Brownian movement is caused by the kinetic energy of the liquid in which the particle is suspended. Gouy [47, 48] states that "the Brownian movement of all the physical phenomena shows us visibly that there is a constant state of internal agitation in liquids even in the absence of all external causes." Many striking experiments have been performed to test the kinetic theory of Brownian motion. Perhaps the most striking experiments are those of Perrin,[49–52] who pointed out that particles of identical size and shape suspended in a liquid distributed themselves according to the same law as is observed by molecules of a gas acted upon by gravity, *i.e.*, the particles are less dense at the surface of a liquid and become more dense as the depth below the surface increases. Perrin counted in a single experiment as many as 13,000 particles at varying depths of 5 μ, 35 μ, 65 μ, and 95 μ below the surface and found that the number of

[46] Burton, E. F., The Physical Properties of Colloidal Solutions, Second Edition, Chapter IV, Longmans, Green and Company, London (1921).

[47] Gouy, M., Note sur le mouvement Brownien, *J. phys.*, 7: 561–564 (1888).

[48] Gouy, M., Sur le mouvement Brownien, *Compt. rend.*, 109: 102–105 (1889).

[49] Perrin, J., L'origine du mouvement Brownien, *Compt. rend.*, 147: 530–546 (1908).

[50] Perrin, J., Grandeur des molécules et charge de l'electron, *Compt. rend.*, 147: 594–596 (1908).

[51] Perrin, J., Mouvement Brownien et réalité moléculaire, *Ann. chim. phys.*, [8] 18: 5–114 (1909).

[52] Perrin, J., Wie wägt man ein Atom? *Z. Elektrochem.*, 15: 269–277 (1909).

particles increased approximately in geometrical progression. As Burton notes, the concentration drops to about one half value in a change of 0.03-mm. difference in level, whereas in the atmosphere a similar decrease would require a difference in level of 6,000 km.

Figure 17 represents the distribution of particles of gamboge and mastic at different levels in their respective sols.

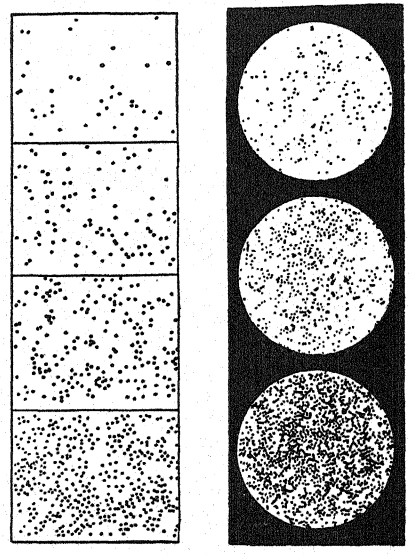


FIG. 17.—A diagrammatic representation of Perrin's experimentation on the distribution of particles in a sol. (After Burton.)

Burton and Currie [53] point out that Perrin's distribution is limited to a very thin surface layer less than 0.1 mm. in depth. Below this layer the distribution of the particles in the sol is uniform. Perhaps these observations are correlated with the concentration of the sol which they investigated. Levine [54] in a mathematical study involving the "atmosphere" of particles under the application of the Debye-Hückel theory finds that a constant distribution (for silver particles of 20-mμ diameter and a specific gravity of 10.5, *i.e.*, a particle weight of 3.9×10^{-14} mg. per particle) is reached at concentrations of 2.1×10^{14} to 5.5×10^{15} particles per cubic centimeter; *i.e.*, constant values are obtained in systems which are as dilute as 0.000004 *normal*, depending on the density of charge (valence) and salt concentration. This may explain why some workers have confirmed Perrin's distribution and others have failed to find it. For silver particles of 20-mμ diameter, Levine calculates limiting values of 8 to 21 mg. silver per cc. If the particles had a specific gravity approximating 1.0 instead of 10.5, then the limiting values of close packing would be reached in the neighborhood of 1–2 mg. per cc. Levine likewise considers the problems of sedimentation equilibrium under centrifugal forces and finds approximately the same limiting values. His calculations are for charged particles; the higher the charge and the lower the salt concentration, the sooner is the limiting value of close packing

[53] Burton, E. F., and Currie, J. E., The Distribution of Colloidal Particles, *Phil. Mag.*, 47: 721–724 (1924).

[54] Levine, S., Problem of the Sedimentation Equilibrium in Colloidal Suspensions, *Proc. Roy. Soc., (London)*, 146A: 597–623 (1934).

reached. Isoelectric particles will pack much more closely, which probably accounts for the flocculation of colloids when the electrical potential at the interface is reduced. These calculations of Levine are of great importance in studies involving sedimentation equilibria and probably account for the failure to observe appreciable sedimentation velocities in the ultracentrifuge when the material which is being investigated possesses an appreciable surface potential. Whether or not the charges on the proteins, due to the zwitterion configuration of proteins at the isoelectric point, influence the sedimentation equilibria is still an undecided point. If the zwitterion charges do influence the sedimentation equilibrium, then the particle weight of proteins calculated from such sedimentation studies may be appreciably in error.

Perrin, finding that the distribution ratios of the various particles at different depths in the surface layer obeyed the gas laws, determined Avogadro's constant for his colloidal sols, and from the equation

$$N = \frac{3}{2}\left(\frac{RT}{W}\right) \tag{14}$$

where N = the number of particles (molecules in the case of a gas) in 1 gram molecule;

W = the mean kinetic energy of a particle (the mean kinetic energy of a molecule in a gas),

he found a value of $N = 70.5 \times 10^{22}$.

Smoluchowski devised an equation based on the displacement of a particle under Brownian movement, due to the kinetic energy of the disperse phase, and Einstein independently developed a similar equation for the motion of small spheres suspended in a liquid medium. Their equations were based on the assumption that the individual particle behaved as a gas molecule and that the gas laws could accordingly be applied, and yielded values for N of 70.5×10^{22} and 65.0×10^{22}, respectively. These values are strikingly in agreement with the values of Millikan, calculated from the direct determination of the electrical charge on ions, *i.e.*, 60.61×10^{22}. We must accordingly note that *colloidal systems obey the gas laws of physical chemistry, providing that each particle of the disperse phase is regarded as behaving as a single molecule.* A gram molecular weight of Perrin's gamboge sol containing 60.61×10^{22} particles would have a "molecular" weight of 30,000,000,000 or approximately 33,000 tons, and this amount of disperse phase would have to be contained in 1 liter of dispersions medium in order to show a normal osmotic pressure and other properties characteristic of a normal solution. Obviously there is no such thing as a molecular weight of 30,000,000,000. The "molecular weight" of colloid micelles is a misnomer, the more appropriate term being particle weight. In biochemical literature molecular weights

of proteins as high as 20,000,000 are recorded. Probably here the more correct term would be particle weight.

Brownian movement undoubtedly tends to stabilize a colloid system. According to Stokes' law of falling bodies,

$$V = \frac{2}{9}\left(\frac{D - d}{\eta}\right) Gr^2 \qquad (15)$$

where V = the velocity of fall;
D = the specific gravity of the falling particle;
d = the specific gravity of the medium through which the fall takes place;
η = the viscosity of the medium;
G = the gravity constant;
r = the radius of the particle.

every substance differing in specific gravity from the medium in which it is suspended will be drawn through that medium by the force of gravity. Applying the above equation to a gold particle 10 mμ in diameter, we find a velocity of fall of approximately 1 cm. per month. Inasmuch as Brownian movement causes a very much greater displacement of the particle than the force of gravity, Brownian movement must be regarded as a stabilizing agent, although, if all convection currents were eliminated and all mechanical vibration controlled, there would be a very slow but inevitable settling of the colloidal particles.

Calculations [55] of the velocity of Brownian movement and the rate of settling, according to Stokes' law, of silver particles having different radii are shown in Table VIII. It will be noted that for particles

TABLE VIII

SHOWING THE RELATIVE VELOCITY DUE TO (*a*) BROWNIAN MOVEMENT AND (*b*) SETTLING ACCORDING TO STOKES' LAW UNDER THE PULL OF GRAVITY, OF SILVER PARTICLES SUSPENDED IN AIR (CALCULATIONS OF BURTON)

Radius of Particle	Velocity of Brownian Movement	Velocity of Sedimentation
Cm. $\times 10^{-7}$	Cm. $\times 10^{-7}$ per Second	Cm. $\times 10^{-7}$ per Second
1000	2,000	1,200,000
500	2,800	300,000
100	6,300	12,000
50	8,900	3,000
10	20,000	120
5	28,000	30
1	63,000	1.2

[55] Burton, E. F., The Physical Properties of Colloidal Solutions, Second Edition, Longmans, Green and Company, New York (1921).

having a diameter greater than 100×10^{-7} cm. (100 mμ) the velocity of sedimentation exceeds the velocity of Brownian movement. However, at 50 mμ the velocity of Brownian movement is approximately three times the velocity of sedimentation, and at the lower limit of the colloid realm it is 60,000 times the velocity of sedimentation.

Inasmuch as the calculations in Table VIII are for silver particles suspended in air, the silver having a specific gravity of 10.5 and air a viscosity of only 1.9×10^{-4}, the sedimentation velocity is very much greater than it would be for a liquid system or for particles of organic materials where the specific gravity approaches the specific gravity of the dispersions medium. If we were dealing with organic particles dispersed in water, the velocity of Brownian movement would exceed the velocity of sedimentation when the radius of the particle was considerably greater than 100 mμ.

Wa. Ostwald [56] has devised a nomograph chart showing the relation of particle size to rate of settling and accordingly to colloidal stability. Anyone knowing the diameter of the suspended particles and their specific gravity can readily determine by means of this chart how long the suspension will be stable, or inversely, knowing the rate of settling and the specific gravity, the diameter of the particles in question can be ascertained. That studies of Brownian movement have more than theoretical interest is illustrated by the papers of Tolman and others,[57-61] who applied the theoretical methods of earlier workers in this field to a study of the efficiency of materials for the production of obscuring smokes to be used in military operations. Figure 18 is a reproduction of a photomicrograph illustrating the paper of Wells and Gerke, taken under conditions where a particle in Brownian movement was allowed to flow slowly across the field.

Size Distribution in Colloidal Systems.—Svedberg and coworkers [62-68] have applied Stokes' law to the determination of the size

[56] Ostwald, Wa., Rechentafel zum Stokes'schen Gesetz, *Kolloid Z.*, 26: 213–215 (1920).

[57] Tolman, R. C., and Vliet, E. B., A Tyndallmeter for the Examination of Disperse Systems, *J. Am. Chem. Soc.*, 41: 297–300 (1919).

[58] Tolman, R. C., Reyerson, L. H., Vliet, E. B., Gerke, R. H., and Brooks, A. P., Relation between the Intensity of Tyndall Beam and Concentration of Suspensions and Smokes, *J. Am. Chem. Soc.*, 41: 300–303 (1919).

[59] Wells, P. V., and Gerke, R. H., An Oscillation Method for Measuring the Size of Ultramicroscopic Particles, *J. Am. Chem. Soc.*, 41: 312–329 (1919).

[60] Tolman, R. C., Gerke, R. H., Brooks, A. P., Herman, A. G., Mulliken, R. S., and Smyth, H. DeW., Relation between Intensity of Tyndall Beam and Size of Particles, *J. Am. Chem. Soc.*, 41: 575–587 (1919).

[61] Tolman, R. C., Reyerson, L. H., Brooks, A. P., and Smyth, H. D., An Electrical Precipitator for Analyzing Smokes, *J. Am. Chem. Soc.*, 41: 587–589 (1919).

[62] Svedberg, The, and Rinde, H., The Ultracentrifuge, a New Instrument for

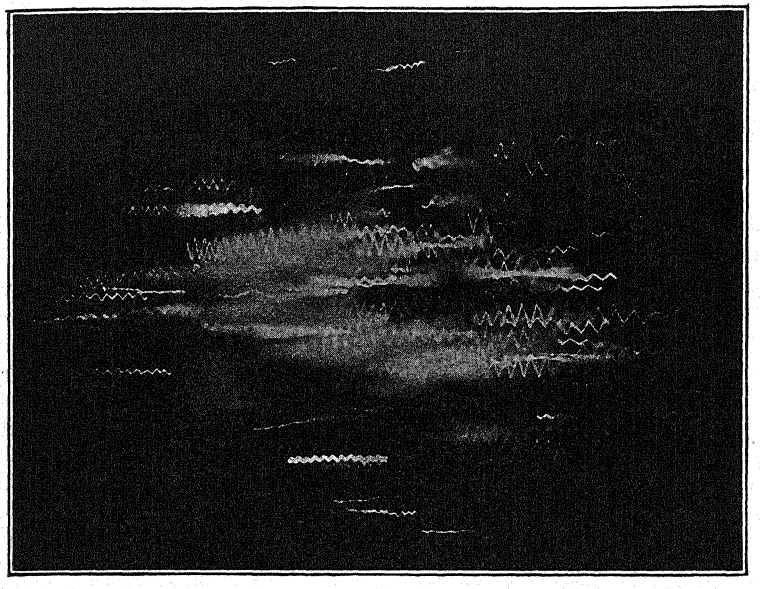


FIG. 18.—Ultramicroscopic photographs showing the vibration due to Brownian movement of colloidal particles moving across the field. Note difference in amplitude as indicating the presence of particles of different sizes. (Photograph, courtesy of Dr. P. V. Wells.)

of colloidal micelles by devising an ultracentrifuge which permits them to study the rate of movement of colloidal particles under a greatly increased gravitational force. Knowing the gravitational force which is applied, they are able to calculate the radius of the particle and accordingly the particle weight of the suspended material.

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the Determination of Size and Distribution of Size of Particle in Amicroscopic Colloids, *J. Am. Chem. Soc.*, 46: 2677–2693 (1924).

[63] Svedberg, The, Zentrifugierung, Diffusion und Sedimentationsgleichgewicht von Kolloiden und hochmolekularen Stoffen, *Kolloid Z., Zsigmondy Festschrift*, 53–64 (1925).

[64] Svedberg, The, and Fahraeus, R., A New Method for the Determination of the Molecular Weight of the Proteins, *J. Am. Chem. Soc.*, 48: 430–438 (1926).

[65] Svedberg, The, and Nichols, J. B., The Application of the Oil Turbine Type of Ultracentrifuge to the Study of the Stability Region of Carbon Monoxide-Hemoglobin, *J. Am. Chem. Soc.*, 49: 2920–2934 (1927).

[66] Svedberg, The, Sedimentation Constants, Molecular Weights, and Isoelectric Points of the Respiratory Proteins, *J. Biol. Chem.*, 103: 311–325 (1933).

[67] Svedberg, The, Sedimentation of Molecules in Centrifugal Fields, *Chem. Rev.*, 14: 1–15 (1934).

[68] Nichols, J. B., The Development of the Ultracentrifuge and its Field of Research, *Colloid Symposium Monograph*, Vol. 6, pp. 287–308, Chemical Catalog Company, New York (1928).

The Svedberg ultracentrifuge has been extensively used in studying the particle size of proteins derived from various biological sources. Because of the large initial cost of the Svedberg instrument, its use has been restricted to very few centers of research. Beams and Pickels [69] have recently devised an ultracentrifuge on an entirely different principle, *i.e.*, a spinning rotor riding on a thin cushion of air and driven by compressed air. With instruments so designed, centrifugal forces as great as 4,000,000 times gravity have been obtained, the possibilities being limited only by the tensile strength of the material from which the rotor is constructed. The initial design of the Beams and Pickels ultracentrifuge has been further modified [70] by using a "quantity" head in which containers can be placed and rotated at speeds up to 30,000 r.p.m., giving forces ranging from 50,000 times gravity at the top to 95,000 times gravity at the bottom. Beams and Snoddy [71] have further modified the instrument by providing an electric drive with rotational speeds up to 60,000 r.p.m. with the rotor in a vacuum, and Wyckoff and Lagsdin [72] and Wyckoff [73] have further modified this instrument. By means of the ultracentrifuge it has been possible to measure sedimentation rates in many colloid systems where such measurements were hitherto impossible, and all proteins so far investigated have been found to sediment from solution in the intense gravitational fields so produced.

Table IX shows the apparent particle weight of a number of the protein systems which have been investigated.

The workers who have used the ultracentrifuge technic almost invariably refer to the apparent particle weights as "molecular weights," and accordingly in recent years an extensive literature has developed regarding the "molecular weights" of proteins, cellulose, and other biochemical compounds as determined by means of the ultracentrifuge. Kruyt [74] has considered this question of terminology in considerable detail, and, because of its importance to a clear understanding of the factors which are involved, it seems desirable to quote at length from Kruyt's statement.

"We have thus come to the discussion of one of the most general

[69] Beams, J. W., and Pickels, E. G., The Production of High Rotational Speeds, *Rev. Sci. Instruments*, 6: 299–308 (1935).

[70] Bauer, J. H., and Pickels, E. G., A High Speed Vacuum Centrifuge Suitable for the Study of Filterable Viruses, *J. Exptl. Med.*, 64: 503–528 (1936).

[71] Beams, J. W., and Snoddy, L. B., The Electrically Driven Ultracentrifuge, *Science*, 85: 185–186 (1937).

[72] Wyckoff, R. W. G., and Lagsdin, J. B., Improvements in the Air-driven Ultracentrifuge for Molecular Sedimentation, *Rev. Sci. Instruments*, 8: 74–77 (1937).

[73] Wyckoff, R. W. G., Quantity Ultracentrifugation with Intense Fields, *Science*, 85: 390–391 (1937).

[74] Kruyt, H. R., The Modern Development of Colloid Chemistry, *Act. IX Congr. Intern. quim. pura y aplicada*, Madrid, April, 1934 (separate 12 pp.).

TABLE IX

SHOWING THE PARTICLE WEIGHTS OF VARIOUS PROTEINS AS DETERMINED BY SVEDBERG AND CO-WORKERS

Protein	Particle Weight	Reference	
Egg albumin	34,500	*J. Am. Chem. Soc.*	48 : 3081 (1926)
Hemocyanin (*Helix pomata*)	5,000,000	*ibid.*	50 : 1399 (1928)
Hemocyanin (*Limulus polyphemus*)	2,040,000	*ibid.*	51 : 539 (1929)
Hemocyanin (*Octopus vulgaris*)	2,000,000	*ibid.*	54 : 4730 (1932)
Phycocyan (*Ceramium rubrum*)	106,000	*ibid.*	50 : 525 (1928)
c-Phycocyan (*Aphanizo menonflos-aquae*)	208,000	*ibid.*	54 : 3998 (1932)
Phycoerythyrin (*Ceramium rubrum*)	208,000	*ibid.*	50 : 525 (1928)
r-Phycoerythyrin (*Ceramium rubrum*)	196,000	*ibid.*	54 : 3998 (1932)
Insulin	35,000	*ibid.*	53 : 2657 (1931)
Edestin	212,000	*ibid.*	51 : 2170 (1929)
Bence Jones protein	35,000	*ibid.*	51 : 3594 (1929)
Amandin (almond)	208,000	*ibid.*	52 : 279 (1930)
Excelsin (Brazil nut)	212,000	*ibid.*	52 : 279 (1930)
Pomelin (orange seed)	210,000	*ibid.*	56 : 1706 (1934)
Legumin (vetch)	208,000	*ibid.*	52 : 3279 (1930)
Casein (Van Slyke and Baker)	75–100,000	*ibid.*	52 : 701 (1930)
Casein (Hammarsten)	375,000	*ibid.*	52 : 241 (1930)
Casein (crude heated to 40°)	188,000	*ibid.*	52 : 701 (1930)
Serum albumin (horse)	67,500	*ibid.*	50 : 3318 (1928)
Serum globulin (horse)	103,000	*ibid.*	50 : 3318 (1928)
Gliadin	34,500	*ibid.*	57 : 946 (1935)
Tobacco virus mosaic	15–20,000,000	*ibid.*	58 : 1863 (1936)
Erythrocruorin (*Arenicola marina*)	2,850,000	*ibid.*	55 : 2834 (1933)
Erythrocruorin (*Lumbricus terrestris*)	2,730,000	*ibid.*	55 : 2834 (1933)
Erythrocruorin (*Planorbis corneus*)	1,341,000	*ibid.*	56 : 1700 (1934)
Erythrocruorin (*Daphnia pulex*)	1,291,000	*ibid.*	56 : 1700 (1934)
Erythrocruorin (*Thyone briareus*)	19,100 to 23,600	*ibid.*	56 : 1700 (1934)
Erythrocruorin (*Myxine glutinosa*)	19,100 to 23,600	*ibid.*	56 : 1700 (1934)
Erythrocruorin (*Petromyzon fluviatilis*)	19,100 to 23,600	*ibid.*	56 : 1700 (1934)
Thyroglobulin	700,000	*Science*	80 : 414 (1934)

problems in colloid chemistry. There is one group of scientists who desire to look upon the micel as a large molecule or as a polyvalent ion. This group is still composed of two groups; those, who believe that we are really dealing with large molecules, molecularly dispersed in the medium, and those, who do not deny a priori the polymolecularity of the particle, but who wish only to discuss its properties as those of a macro molecule. On the other hand there are the colloid chemists, who assume colloids to be intermediate between molecules and coarse aggregates, consequently polymolecular particles, and who want to derive the properties from those studied on macro walls. Though there will be no doubt, after what I have just said, concerning

the class to which I belong myself, I want to do justice to both principles.

"First of all I want to state, that the controversy is absolutely unreal from the kinetic point of view. The identity of Brownian movement and the molecular heat movement is of course conclusive in this respect. However, it is sensible to realise the consequences of this statement. When we measure osmotic pressure, change of freezing or boiling points etc., *we count particles*; whether these particles are monomolecular or polymolecular is of no importance. When 61×10^{22} independent particles of what structure whatever are present in 22.4 L of water, the osmotic pressure is 1 atmosphere, the depression of the freezing point 1.8°/22.4, etc. These laws are generally applied to true solutions and therefore we are accustomed to identify the thus measured number of particles with that of the molecules present. However, we must keep in mind, that this is an arbitrary identification, what we really do is only count kinetically active particles. A similar conclusion holds for sedimentation experiments (of course centrifuging experiments included); in that way we measure the particle's radius, whether it is a single molecule or a polymolecular micel. Only when we know in an independent way the real molecular weight, are we able to draw a conclusion from these *two* independent values, but without the latter, kinetic measurements give only *micellar* numbers and *micellar* weights.

"In the second place I like to acknowledge, that a colloidal particle, with its electric double layer and its atmosphere of orientated water dipoles of hydration *may* be considered as a polyvalent ion. The only question to be put is, whether this is fruitful or not; could it be fruitful then there is no fundamental objection. What we always try in science is to develop our insight on the basis of previously acquired knowledge. So, if we had a fundamental knowledge of monovalent, divalent, threevalent, quadrivalent . . . polyvalent ions in very dilute solutions (in molarity, *i.e.* as a fraction of 61×10^{22} particles per L; colloidal solutions are extremely dilute, though—another complication!—the volume occupied by the dissolved substance is very large), I say, if we really had such knowledge we could extend it successfully to the colloidal particles, which have always from 10 to hundreds and thousands of ionogenic spots on their surface. However, we lack such knowledge, the Debye-Hückel theory has extended our concepts of strong electrolytes considerably, but other electrolytes than those of the 1.1 type, at best of the 1.2 type, we are at a loss to give a satisfactory quantitative explanation of conditions. Thus, what can be the use of looking upon a colloid particle as a giant polyvalent ion, other than as a metaphore? It would not be wrong, but it is useless. For the present it must be much more promising to refer to the knowledge acquired on the electric phenomena at walls, capillary and

electrokinetic phenomena. I should not like to say that *these* are entirely understood, nor that we do not need much more information about them, but we can compare them directly to colloid phenomena without risky extrapolation. I can imagine very well, that the day will come, when ionic theories and electrokinetic theories at phase boundaries will come to one single unity and that the controversy I am discussing at this moment will fall away, but the fruitful task for science is always to put problems on the basis of present knowledge, to explain phenomena on the basis of sound experience, and to choose working hypotheses in harmony with what we really can perceive.

"Moreover, we must take care to bring colloid chemistry more and more on the level of quantitatively explaining physical chemistry. We cannot content ourselves nor with mere comparisons nor with morphological descriptions. Colloids form an entity, not that the knowledge of the constituting molecules should be neglected, nor that colloids should be interpreted as mere walls in water; nobody should deny that there must be a continuous relation between colloidal systems and true solutions, but on the other hand a similar continuity must exist between colloidal systems and coarse suspensions of undeniable polymolecularity, and premature and superficial identifications do not promote real motion."

The author of this volume is in complete accord with the reasoning of Professor Kruyt, and in the discussions which follow, dealing with the behavior of colloid micelles as "molecules" or "ions," it will be understood that, although the individual colloidal particle may behave as an individual "molecule" or an individual "ion," nevertheless the mass of that "molecule" or "ion" may not necessarily be a fixed quantity but may vary in many instances over a considerable range.

Nichols, Kraemer, and Bailey [75] studied in the ultracentrifuge the sedimentation velocity of ferric oxide sols prepared by various methods and aged for various lengths of time. Figure 19, taken from their paper, shows the effect of the concentration of the $FeCl_3$ hydrolyzed to produce the Fe_2O_3 on the particle weight distribution. This curve is inserted to show that such sols do not consist of uniform-sized particles but rather that the size of the particles in a given sol forms a typical distribution curve which may in one instance have a steep slope, in other instances be greatly flattened, and in still others be skewed in one direction.

Odén [76] has discussed general methods of determining particle size in soils, and these methods are by and large applicable to colloidal systems which show an appreciable velocity of settling. Odén im-

[75] Nichols, J. B., Kraemer, E. O., and Bailey, E. D., The Particle Size and Constitution of Colloidal Ferric Oxide, *J. Phys. Chem.*, 36: 326–339 (1932).

[76] Odén, S., Methods to Determine the Size-distribution of Soil-particles, *Proc. Fourth Intern. Soil Science Conference*, Sect. I-A, No. 5 (separate 32 pp.) (1924).

mersed one pan of an analytical balance in the suspension under investigation. As the particles settled to the bottom as a function of time the weight on this pan of the balance increased, and accordingly the

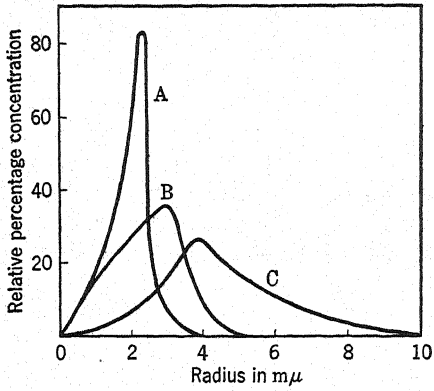


FIG. 19.—Effect of concentration during hydrolysis on the particle size distribution curves of ferric oxide hydrosols prepared from different concentrations of ferric chloride (all sols hydrolyzed for 1 hour at boiling temperature). Curve *A*, 0.003 *M* $FeCl_3$, mean radius, 2.0 mμ; curve *B*, 0.005 *M* $FeCl_3$, mean radius, 2.6 mμ; curve *C*, 0.037 *M* $FeCl_3$, mean radius 4.4 mμ. (Data of Kraemer.)

rate of settling and the mass of particles settling in a unit time could be determined by appropriate weighing technics. Markley [77] has applied this method to the problem of the distribution of particle size in wheat flours with gratifying results.

Other devices which have proved useful are the Kelly [78] sedimentation tube where the particles settling from the suspension have a greater density than the liquid in which they are suspended, and the Stamm [79] tube designed for use with emulsions where the suspended particle has a lower density than the liquid in which it is suspended. Both these tubes are variations of Wiegner's [80] original sedimentation tube. The sedimentation tube method has proved of great value in many industrial laboratories. For technic, operating, and calculating

[77] Markley, M. C., Flour Particle Size by the Sedimentation Method, *Cereal Chem.*, 11: 654–660 (1934).

[78] Kelly, W. J., Determination of Distribution of Particle Size, Colloid Symposium Monograph, Vol. II, pp. 29–36, Chemical Catalog Company, New York (1925).

[79] Stamm, A. J., A New Method for the Determination of the Distribution of Size of Particles in Emulsions, Colloid Symposium Monograph, Vol. II, pp 70–79, Chemical Catalog Company, New York (1925).

[80] Wiegner, G., Über eine neue Methode der Schlämmanalyse, *Landw. Versuchsstat*, 91: 41–79 (1918).

the results, the authors already noted should be consulted, and in addition the paper by Duncombe and Withrow [81] should be of value.

Osmotic Pressure of Colloid Systems.—As already noted, the gas laws can be applied to colloidal systems, providing each particle is considered as having the same kinetic energy as a molecule. Accordingly, the fundamental equation

$$PV = NRT \tag{16}$$

should hold for colloid systems, but as we have seen from Kruyt's discussion cited in the preceding section, such application of physicochemical laws does not necessarily mean that the particles which are "counted" are in reality molecules.

A gram molecule of a gas occupies approximately 22.4 liters volume at 0° and 760 mm. pressure. Inasmuch as the volume is inversely proportional to the pressure, a gram molecule of a gas would occupy 1 liter volume at 0° and approximately 22.4 atmospheres pressure. Solutions obey the gas laws provided that the volume in the above equation is the volume of the solvent and not the volume of the solvent plus solute. In a later section we will have occasion to discuss the osmotic pressure phenomenon exhibited by crystalloids and its relation to biological phenomena. At present we are only concerned with osmotic pressure exhibited by colloidal systems.

As noted in the section on Brownian movement, Perrin's gamboge sols had an apparent "molecular weight" of 30,000,000,000. Accordingly, 30,000,000 kg. of the gamboge particles of the size studied by Perrin, if suspended in 1 liter of water, should give a sol possessing a normal osmotic pressure, *i.e.*, 22.4 atmospheres. Similarly, if a red gold sol could be prepared containing approximately 300 pounds of red gold in 1 liter of water, we would observe an osmotic pressure of approximately 22.4 atmospheres. Red gold sols more concentrated than 1 gram of disperse phase in 1 liter of dispersions medium are rarely met with. Such sols as are obtainable show little or no osmotic pressure, and from the above considerations this is to be expected, inasmuch as a red gold sol containing only 1 gram of disperse phase in a liter of solvent should have an osmotic pressure only slightly greater than 0.1 mm. Hg, providing each particle possessed the kinetic energy of a molecule. Inasmuch as osmotic pressure is the driving force of diffusion, it is easy to understand why the disperse phase in most colloid systems possesses such a low diffusion coefficient.

The osmotic pressure of solutions of sols of biocolloids, such as proteins, is extremely low even when the biocolloid is present in mono-

[81] Duncombe, C. G., and Withrow, J. R., The Kelly Tube and the Sedimentation of Portland Cement, *J. Phys. Chem.*, 36: 31–51 (1932).

molecular condition. Sörensen [82] made an elaborate study of the osmotic pressure of egg-albumin solutions, and he has definitely proved that the egg albumin is present in monomolecular solution. As already noted in the earlier pages, this does not necessarily exclude egg-albumin solutions from being typical colloidal sols. The author has no doubt but that they are typical colloidal sols, the micelles of which are individual egg-albumin molecules of a size sufficient (together with water of hydration) to bring the size of the molecule well within the limits of the colloidal realm. In Sörensen's studies of the osmotic pressure of egg-albumin sols, the pressure was measured against a water manometer. Directly measured osmotic pressures as high as 86 cm. of water pressure were observed for an egg-albumin solution containing 22.66 grams of egg-albumin hydrate in 100 grams of water. Such values probably approach the upper limit of osmotic pressure values for lyophilic colloid systems. For most lyophilic systems much smaller values than these will be encountered, in many instances the osmotic pressure being only 1 or 2 cm. of water pressure for similar concentrations, and for many lyophobic systems the value for osmotic pressure may be so small as not to be measurable.

Dobry [83] has devised a very sensitive apparatus for measuring the osmotic pressure in colloidal systems and claims that an accuracy of 0.1 to 0.2-mm. rise in pressure of solvent can be obtained with the apparatus. He finds by this method a "molecular weight" of 111,000 for nitrocellulose.

Charged colloidal particles likewise can be considered as ions. Bikerman,[84] in a theoretical paper, considers colloidal particles as ions but states that the colloidal micelles present in sols or gels contribute an insignificant fraction to the electrical conductivity of such systems.

Diffusion in Colloid Systems.—Graham's early distinction between colloidal and crystalloidal systems was largely based upon the pronounced difference in the rate of diffusion in the two instances. Graham found that if various materials were placed in bags of parchment paper or animal membrane, such as dried bladder or goldbeater's skin, the various substances could be divided into two general classes: (1) those that readily passed through the parchment paper or animal membranes, and (2) those that failed to pass through such membranes. The former he called crystalloids, the latter colloids; and non-diffusion

[82] Sörensen, S. P. L., Studies on Proteins, Chap. V, *Compt. rend. trav. lab. Carlsberg*, Vol. 12, 1915–1917.

[83] Dobry, A., Appareils pour la mesure de très petites pressions osmotiques dans les solutions colloidales, *J. chim. phys.*, 32: 46–49; Pression osmotique des solutions de nitrocellulose, *ibid.*, 32: 50–57 (1935).

[84] Bikerman, J. J., La conductibilité électrique des solutions colloidales, *J. chim. phys.*, 32: 460–465 (1935).

through a membrane was taken as the criterion of the colloid state. In regard to the diffusion coefficient, he states, "It is easy to see that such a constant must enter into all chronic phenomena of physiology and that it holds a place in vital science not unlike the time of the falling of heavy bodies in the physics of gravitation."

The separation of colloids from crystalloids by the process of diffusion is called *dialysis*. Various membranes may be employed in dialysis. The more commonly used membranes are parchment paper, goldbeater's skin, animal membranes, and collodion membranes. Recently pure cellulose membranes in the form of cellophane sheets and tubes have replaced to a considerable extent collodion membranes for laboratory use.

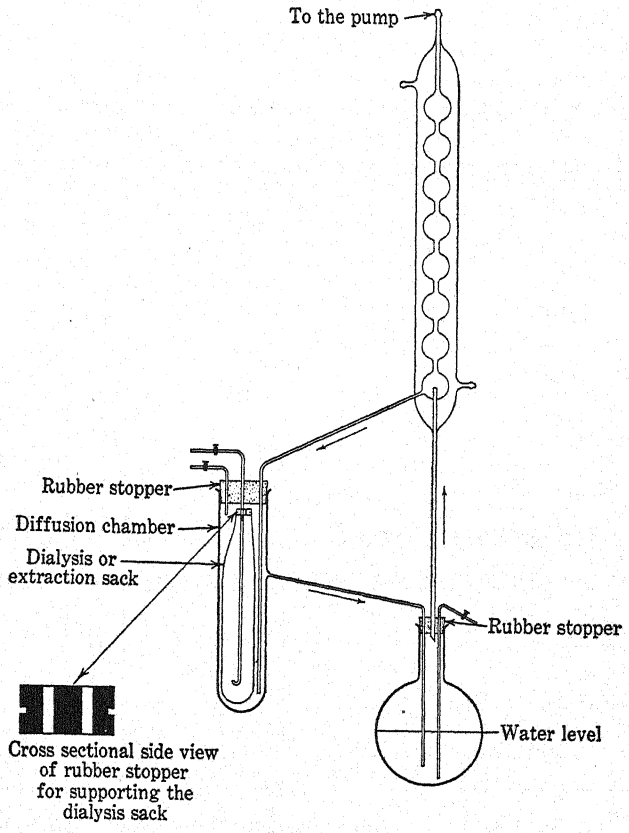


FIG. 20.—Apparatus for the continuous dialysis of biocolloids operating at reduced pressure with a constant volume of liquid. (After Hanke and Koessler.)

Hanke and Koessler[85] devised an apparatus for continuous dialysis of hydrosols or hydrogels against water at constant volume. This apparatus, shown in Fig. 20, is extremely useful in many biochemical operations, particularly where one desires to ascertain the ratio of colloid to total solids.

[85] Hanke, M. T., and Koessler, K. K., A Continuous Dialysis or Extraction Apparatus Which Operates at Reduced Pressure with a Constant Volume of Liquid, *J. Biol. Chem.*, 66: 495–499 (1926).

It is obvious that, if true solutions grade almost imperceptibly into colloidal systems and colloidal systems grade equally imperceptibly into coarse suspensions, there is no sharp line of demarcation, even with respect to rate of diffusion, between true solutions and colloid systems. We would accordingly expect to find true solutions which would have a low diffusion coefficient and pass extremely slowly through a dialyzing membrane, and we should likewise expect to find colloidal sols possessing a measurable rate of diffusion and likewise passing slowly through dialyzing membranes. It is therefore not surprising to find that egg albumin in appreciable amount may dialyze through a membrane. Membranes can, however, be prepared which are sufficiently dense to retain the egg-albumin molecules. Table X lists certain more or less familiar materials with the corresponding coefficients of diffusion.

TABLE X

SHOWING THE COEFFICIENTS OF DIFFUSION OF CERTAIN COMMON CRYSTALLOIDAL AND COLLOIDAL MATERIALS

	Coefficient of Diffusion	Temperature, ° C.
Nitric acid	2.10	20.0
Sodium chloride	1.04	20.0
Urea	0.81	7.5
Copper sulfate	0.47	17.0
Cane sugar	0.31	9.0
Nuclear gold sol (Svedberg)	0.27	11.7
Pepsin	0.070	18.0
Rennin	0.066	18.0
Egg albumin	0.059	18.0
Ovomucoid	0.044	18.0
Emulsin	0.036	18.0
Diphtheria toxin	0.014	12.0
Diphtheria antitoxin	0.0015	12.0
Tetanolysin	0.037	12.0
Antitetanolysin	0.0021	12.0

It will be noted that the coefficient of diffusion for sucrose lies very close to the coefficient of diffusion for the amicroscopic gold hydrosol. Accordingly, we should expect a very appreciable amount of amicroscopic gold hydrosol to pass through a membrane, and this is what actually takes place. On the other hand, the extremely low coefficient of diffusion for the antitetanolysin would mean that practically none of this material would diffuse, and this is what is actually observed. The diffusion velocity is inversely proportional to the size of the particle, and an equation may be written

$$\Delta r = K \tag{17}$$

where Δ = the diffusion coefficient;
r = the radius of the particle;
K = a constant.

According to this equation the diffusion velocities of two substances are inversely proportional to the radii of those substances, so that if we have one material, the diffusion coefficient of which is known, and we know the radius of the diffusing particles, we can determine either the diffusion coefficient or the radius of an unknown particle, provided that one or the other is known, according to the equation

$$\frac{\Delta_1}{\Delta_2} = \frac{r_2}{r_1} \tag{18}$$

Exner has shown that for gases

$$\Delta\sqrt{M} = K \tag{19}$$

where

M = the molecular weight

and Öholm has shown that this holds for dilute solutions of non-electrolytes. We have already seen that colloid systems obey the gas laws, provided that each individual particle is regarded as a molecule. Accordingly, we can calculate the particle weight by equation (19), and it has been found that the particle weight, or so-called molecular weight, for egg albumin, hemoglobin, etc., calculated by this method, agrees fairly well with values obtained by other and independent physical measurements.

Anson and Northrop [86] discuss the technic for making determinations of the rate of diffusion of proteins and enzymes and from such data the calculation of the "molecular weight." In a comparison of their results with other results obtained by the direct osmotic or sedimentation methods, they find that in general the diffusion values are somewhat high, possibly owing to the hydration of the particles or perhaps to the fact that the particles may be non-spherical.

Svedberg [87] has used the equation

$$\Delta = \frac{RT}{N} \cdot \frac{1}{6\pi\eta r} \tag{20}$$

in order to test the behavior of colloids in relation to their kinetic energy. He found for a nuclear gold sol, $r = 1.29\ m\mu$. By an independent method not based on the diffusion velocity, a value of $r = 1.33\ m\mu$ was obtained. When the value of $1.33\ m\mu$ was taken as the value of r,

[86] Anson, M. L., and Northrop, J. H., The Calibration of Diffusion Membranes and the Calculation of Molecular Volumes from Diffusion Coefficients, *J. Gen. Physiol.*, 20: 575–588 (1937).

[87] Svedberg, The, Colloid Chemistry, Second Edition, p. 96, Chemical Catalog Company, New York (1928).

and N was calculated from equation (20), an Avogadro constant of 58×10^{22} was obtained, which agrees very well with the generally accepted values.

Certain special applications of phenomena involving diffusion in colloid gels will be taken up later.

PERSTILLATION AND PERVAPORATION.—Kober [88] described an important technic which appears to have been rather generally overlooked. He notes that, if one encloses liquid in a collodion (probably cellophane would be preferable) bag and suspends such a bag over a free flame or an electric heater, one has in reality a ball of water suspended in air with evaporation possible on all surfaces. Under these conditions it is practically impossible to raise the liquid inside of the bag to the boiling point, and evaporation is extremely rapid. This phenomenon he calls pervaporation. If the liquid contains both crystalloids and colloids, the crystalloids will diffuse through the membrane with the water and remain, after the evaporation of the water, on the outside of the membrane in crystal form completely free from colloidal contaminants which will remain inside of the membrane.

FILTRATION AND ULTRAFILTRATION OF COLLOID SYSTEMS.—As a rule, colloid sols pass unchanged through filter paper or through the porcelain filters of the bacteriological laboratory. The pores in the usual filter paper are approximately 2 to 5 μ in diameter, and the pores of the finer Berkefeld and Chamberlain porcelain filters range in size from 0.2 to 0.6 μ. It is accordingly understandable why colloid sols should pass through such filters, inasmuch as the size of the colloidal particle is considerably less than the size of the opening through which the particle must pass. Matter in mass can, therefore, be separated from colloidal sols by passage through a bacterial filter.

Ultrafilters, however, have been devised with pores sufficiently fine to retain the disperse phase and allow only the dispersions medium and its crystalloidal solutes to pass through. This process of separation is known as ultrafiltration. Ultrafilters are invariably colloid gels, so that ultrafiltration may be defined as filtration of a sol through a gel.

Various types of ultrafilters have been devised. Those commonly used are membranes of collodion or gelatin, although rubber membranes have been used in some instances. The technic of ultrafiltration is largely due to the pioneer studies of Bechhold,[89,90] who used filter paper and cloth as the supporting membrane for collodion or gelatin films. The paper or cloth was coated with collodion or with gelatin,

[88] Kober, P. A., Pervaporation, Perstillation, and Percrystallization, *J. Am. Chem. Soc.*, 39: 944–948 (1917).

[89] Bechhold, H., Kolloidstudien mit der Filtrationsmethode, *Z. physik. Chem.*, 60: 257–318 (1907).

[90] Bechhold, H., Durchlässigkeit von Ultrafiltern, *Z. physik. Chem.*, 64: 328–342 (1908).

the gelatin subsequently being hardened by immersion in a dilute solution of formaldehyde.

The direct measurement of osmotic pressure of crystalloidal solutions is carried out by making use of a membrane of copper ferricyanide precipitated in the pores of a porous porcelain vessel. Such a membrane can be made which is truly semi-permeable and which will allow the passage of water molecules but retard the passage of most crystalloidal solutions, such as solutions of dextrose, sucrose, etc. An ultrafilter differs only in degree from such a membrane, the pores of an ultrafilter being larger in diameter than the pores of a truly semi-permeable membrane. All gradations of pore size of ultrafilters can be obtained between the ranges of a copper ferricyanide membrane and ordinary filter paper, and numerous papers have been published, dealing with the technic for preparing such graded ultrafilters. One of the earlier papers is that by Schoep,[91] who points out that almost any degree of permeability of collodion membranes can be obtained by mixing varying proportions of glycerol and castor oil with the collodion solutions. If it is desirable to conduct ultrafiltrations under high pressure, the membranes may be precipitated in a porous porcelain support. When so prepared, pressures up to 100 atmospheres may be applied to induce filtration.

Farmer [92] and Eggerth [93] both studied the procedure for producing ultrafilters of varying porosity. Eggerth controlled porosity by varying the ratio of alcohol to ether in the solvent which was used to dissolve the collodion. He designated the porosity of the collodion mem-

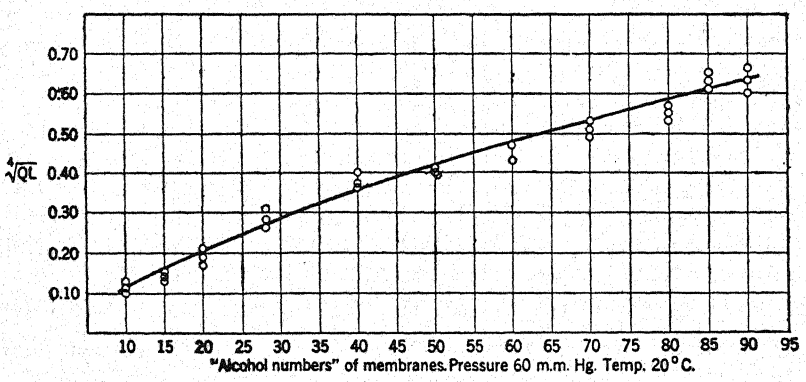


FIG. 21.—Showing the relation between permeability of ultrafilters and Eggerth's "alcohol numbers." (Data of Eggerth.)

[91] Schoep, Alfred, Über ein neues Ultrafilter, *Kolloid Z.*, 8: 80–87 (1911).

[92] Farmer, C. J., A Method for the Preparation of Uniform Collodion Membranes for Dialysis, *J. Biol. Chem.*, 32: 447–453 (1917).

[93] Eggerth, A. H., The Preparation and Standardization of Collodion Membranes, *J. Biol. Chem.*, 48: 203–221 (1921).

brane according to its "alcohol number," *i.e.*, the percentage of alcohol in the solvent used to dissolve the collodion. Figure 21 shows the relationship between alcohol number and the diameter of the pores. The diameter is proportional to $\sqrt[4]{QL}$, where Q is the volume of liquid passing through the filter in unit time, and L is the thickness of the membrane.

Duclaux and Errera,[94] studied the mechanism of ultrafiltration and point out that the velocity of flow of liquid through the pores of the membrane is inversely proportional to the viscosity of the liquid and directly proportional to the pressure, the membrane behaving exactly like a bundle of fine capillary tubes. On the other hand, Brinkman and Szent-Györgyi [95] point out that surface tension or interfacial tension may profoundly alter the characteristics of a collodion ultrafilter. They found that when a hemoglobin sol was placed in a collodion bag and subjected to a pressure of 3 atmospheres, the hemoglobin was completely retained and only water passed the membrane. If, however, a dilute solution of sodium oleate was first passed through the filter, the membrane became permeable to the hemoglobin. The hemoglobin which passed the sodium oleate-treated membrane would not subsequently pass through a second untreated membrane. Accordingly, the size of the micelles in the hemoglobin sol which had passed through the treated membrane had not been affected by the sodium oleate. The size of the pores in the treated membrane had not been altered, as was shown by rate of water filtration through the pores before and after the sodium oleate treatment. Sodium caproate, which has little or no effect on interfacial tension, did not alter the properties of the collodion membrane. Sodium linoleate, sodium glycocholate, digitonin, α-monooleinglycerol ester, and Witte's peptone, which lower the interfacial tension in varying degrees, affect the permeability of the collodion membrane in the same ratio as they affect interfacial tension.

In a second paper [96] they studied the effects of alkaloids and purine bases on permeability of ultrafilters, using atropine, pilocarpine, caffeine, strychnine, quinine, and morphine. A change in the permeability of the collodion membrane toward hemoglobin was noted. Biologically inactive codeine did not alter the permeability; neither did cocaine nor novococaine. These results are very striking, and, as Brinkman and

[94] Duclaux, J., and Errera, J., Le mécanisme de l'ultra-filtration, *Rev. gén. colloïdes*, 2: 130–139 (1924).

[95] Brinkman, R., and Szent-Györgyi, A. v., Studien über die physikalisch-chemischen Grundlagen der vitalen Permeabilität. I. Die Wirkung kapillaraktiver Stoffe auf die Permeabilität von Kollodiummembranen, *Biochem. Z.*, 139: 261–269 (1923).

[96] Brinkman, R., and Szent-Györgyi, A. v., Studien über die physikalisch-chemischen Grundlagen der vitalen Permeabilität. II. Die Wirkung von Alkaloiden und Purinbasen auf die Permeabilität von Kolloidiummembranen, *Biochem. Z.*, 139: 270–273 (1923)

Szent-Györgyi suggest, may have a fundamental bearing on reactions which take place in living organisms.

Varney and Bronfenbrenner [97] studied Kendall's "K" medium in which Kendall claims to secure a "filterable form" of *B. typhosus*. They grew the bacteria on agar slants, suspended them in sterile "K" medium, and filtered it at once. The bacteria in the "K" medium passed through the filter; those in broth did not. If sterile "K" medium was first passed through the filter and then the organisms in broth or saline filtered through the same ultrafilter, the organisms passed through. This is another illustration of the importance of surface relationships in ultrafiltration or in ordinary filtration.

Lundsgaard and Holbøll [98] have likewise studied the preparation, standardization, and calibration of collodion membranes. They use a rather novel method of determining porosity. A glucose solution of known concentration is placed in a collodion bag or tube and allowed to dialyze against pure water for a definite interval of time. Analysis for glucose in the inner and outer liquids is made at intervals. When the volume of liquid inside the membrane is equal to the volume of liquid outside the membrane, the following equation can be applied

$$\frac{dx}{dt} = \Delta\left(\frac{K}{2} - x\right) \tag{21}$$

where K = the original concentration of glucose in the inner liquid;
x = the increase in glucose concentration in the outer liquid in time, t;
Δ = diffusion coefficient of glucose for a given membrane.
Accordingly,

$$\Delta = \frac{1}{t}\log\frac{K}{K - 2x} \tag{22}$$

Lundsgaard and Holbøll found from experiment that the original concentration of glucose (within limits ranging from 0.1 per cent to 0.3 per cent concentration) made no difference in the value obtained for the diffusion coefficient. They prepared membranes of definite porosity by coating glass tubes with collodion and then immersing the collodion membrane, when partly dry, in alcohol of known concentration for a fixed period of time, followed by immersion in water. When alcohol of 70 per cent concentration was employed, the diffusion coefficient of glucose through the membrane ranged from $\Delta = 0.0133$ to $\Delta = 0.0122$ as maximum and minimum ranges (eight membranes

[97] Varney, P. L., and Bronfenbrenner, J., The Effect of "K" Medium on the Filterability of Bacteria, *Proc. Soc. Exptl. Biol. Med.*, 29: 804–806 (1932).

[98] Lundsgaard, C., and Holbøll, S. A., Investigations into and the Standardization and Calibration of Collodion Membranes I., *J. Biol. Chem.*, 68: 439–456 (1926).

tested). When 80 per cent alcohol was used, the maximum and minimum ranges were $\Delta = 0.0097$ to $\Delta = 0.0093$; and when 90 per cent alcohol was used, similar values were $\Delta = 0.0058$ to $\Delta = 0.0057$. They state that the diffusion coefficient is not altered after the membranes have been aged for one week. Hitchcock [99] applied the viscosity equation to the measurement of the size of pores in collodion membranes. In the membranes that he worked with, the radius of the pores ranged from 20.8 mμ to 2.7 mμ. He points out that in the former case there are 70 billion capillary tubes per square centimeter and in the latter case 2700 billion capillary tubes per square centimeter.

Bechhold and Heymann [100] used ultrafiltration to concentrate gelatin sols, the dispersions medium passing through and the gelatin micelles being retained on the filter. In this way the ash content was greatly reduced. Using membranes of varying porosity they state that they were able to separate gelatin into two fractions, one of which passed through the more porous membrane.

It is often desirable to test the porosity of a membrane by more or less qualitative methods. This can be done by making use of a series of colloidal sols ranging from a Prussian blue sol to a truly crystalloidal solution. The following list of materials has been suggested by various workers as offering a graded series of particle size.

Prussian blue > Bredig's platinum sol > casein in milk > As_2S_3 sol > Zsigmondy's red gold sol > 1 per cent gelatin sol > hemoglobin sol > litmus > nuclear gold sol > crystalloids.

Krueger and Ritter [101] and Bauer and Hughes [102] give detailed directions for preparing graded ultrafiltration membranes and standardizing them for the study of biological problems. Table XI taken from the paper of Bauer and Pickels [103] lists the approximate particle sizes of various viruses as determined by ultrafiltration technic. The ultrafiltration apparatus described by Bauer and Hughes has been found to be very serviceable in the author's laboratories. Other papers that may be advantageously consulted are that by Ferry,[104] which contains a detailed literature review of the various types of ultrafilters, their

[99] Hitchcock, D. I., The Size of Pores in Collodion Membranes, *J. Gen. Physiol.*, 9: 755–762 (1926).

[100] Bechhold, H., and Heymann, E., Konzentrierung und Reinigung von Lösungen hydrophiler Kolloide, *Biochem. Z.*, 171: 33–39 (1926).

[101] Krueger, A. P., and Ritter, R. C., The Preparation of a Graded Series of Ultrafilters and Measurement of their Pore Sizes, *J. Gen. Physiol.*, 13: 409–419 (1930).

[102] Bauer, J. H., and Hughes, T. P., The Preparation of the Graded Collodion Membranes of Elford and their Use in the Study of Filterable Viruses, *J. Gen. Physiol.*, 18: 143–162 (1934).

[103] Bauer, J. H., and Pickels, E. G., A High Speed Vacuum Centrifuge Suitable for the Study of Filterable Viruses, *J. Exptl. Med.*, 64: 503–528 (1936).

[104] Ferry, J. D., Ultrafilter Membranes and Ultrafiltration, *Chem. Rev.*, 18: 373–455 (1936).

structure, and their application to specific problems (296 papers are cited), and that by Höber,[105] which contains data on membranes of

TABLE XI

APPROXIMATE PARTICLE SIZE OF VIRUSES AS DETERMINED BY FILTRATION THROUGH GRADED COLLODION MEMBRANES

(By permission, *J.. Exptl. Med.*)

Virus	Estimated Particle Size, Millimicrons	Virus	Estimated Particle Size, Millimicrons
Vaccinia	125–175	Vesicular stomatitis	70–100
Canary pox	125–175	Fowl plague	60– 90
Lymphogranuloma inguinale	125–175	Rift Valley fever	23– 35
Rous sarcoma 1	100–150	Equine encephalomyelitis	20– 30
Ectromelia	100–150	St. Louis encephalitis	20– 30
Pseudorabies	100–150	Yellow fever	17– 25
Herpes	100–150	Louping ill	15– 20
Borna disease	85–125	Poliomyelitis	10– 15
Influenza, swine and human	80–120	Foot-and-mouth disease	8– 12

various pore sizes with particular reference to the rate at which ions may pass across membranes.

It is sometimes impossible to use ultrafiltration procedure, owing to the fact that collodion or gelatin membranes are altered by the liquid which one desires to filter. Since such membranes are colloid gels, they would be affected by hot solutions and by solutions which are definitely acid or alkaline. Manning[106] prepared metallic ultrafilters by plating nickel on phosphobronze or nickel gauze under such conditions that the plated nickel is deposited in a porous state. His paper gives directions for current density and concentration of solutions which should be employed. Such ultrafilters can be used with organic solvents and are not affected by relatively high temperatures.

An important contribution to the literature of ultrafiltration is the observation by Kramer,[107] who emphasizes the role of the charge on the colloid membrane in studies of filtration. Kramer was interested particularly in the nature of the so-called filterable microorganisms and viruses which pass unaltered through the porcelain filters of the

[105] Höber, R., Membrane Permeability to Solutes in its Relations to Cellular Physiology, *Physiol. Rev.*, 16: 52–102 (1936).

[106] Manning, John, The Preparation of Nickel Membranes for Ultrafiltration, *J. Chem. Soc.*, May, 1926, 1127–1132.

[107] Kramer, S. P., Bacterial Filters, *J. Gen. Physiol.*, 9: 811–812 (1926).

bacteriological laboratory. He points out that all Berkefeld and Pasteur-Chamberlain filters are made from siliceous materials and consequently possess a negative charge. He found that colloidal dyestuffs possessing a positive charge would not pass such filters, whereas those which were negatively charged passed readily. For example, Victoria blue (+) was retained in a Berkefeld filter, whereas Congo red (−) readily passed through. He, accordingly, prepared filters similar to the Berkefeld filter but possessing a positive charge. Using plaster of Paris ($CaSO_4$) filters, he found no differentiation between acid and basic dyestuffs. When, however, a small amount (± 5 per cent) of calcium carbonate was added to the plaster of Paris prior to forming the filter, he obtained positively charged filters which retained the Congo red (−) sols and allowed the Victoria blue (+) to pass through, thus reversing the retainability of the Berkefeld filter. A still more striking observation of Kramer is the fact that the bacteriophage of *Staphylococcus aureus*, the *Vibrio percolans* of Mudd, vaccine virus, and rabies virus are retained by the positively charged plaster of Paris filters, whereas they readily pass through the ordinary bacteriological filters. As we shall see later in a discussion of the electrical properties of colloids, the retention is probably due to the mutual precipitation of oppositely charged micelles. A further illustration is found in the observations of Mulvania [108] that the virus of tobacco mosaic will pass through a collodion ultrafilter normally impermeable to the virus, provided that the *p*H of the virus solution is changed to the acid side of the virus isoelectric point.

VIVIDIFFUSION.—Abel, Rowntree, and Turner [109] applied the methods of dialysis and ultrafiltration to a study of the crystalloids present in the blood stream. Figure 22 shows the apparatus which they devised. Using a glass cylinder open at both ends, they inserted within the cylinder a number of collodion tubes. These tubes were connected in series by glass U-tubes, together with in-flow and out-flow tubes which passed through rubber stoppers closing the ends of the glass cylinder. With the tubes in place and filled with physiological salt solution and surrounded with physiological salt solution, the in-flow tube was connected to the carotid artery of the experimental animal, the out-flow tube being connected to the femoral vein. Hirudin (leach extract, an anti-blood-coagulant) is injected into the blood before it passes from the carotid artery into the system of collodion tubes, thus preventing the blood from coagulating when it comes in contact with the glass connecting tubes. The liquid surrounding the collodion tubes

[108] Mulvania, M., Studies on the Nature of the Virus of Tobacco Mosaic, *Phytopath.*, 16: 853–871 (1926).

[109] Abel, J. J., Rowntree, L. G., and Turner, B. B., On the Removal of Diffusible Substances from the Circulating Blood of Living Animals by Dialysis, *J. Pharmacol.*, 5: 275–316 (1914).

is kept at blood temperature by means of a thermostat. Thus, it is possible to pass blood from the heart of an animal outside of the body through a dialyzing and ultrafiltration apparatus, back through the venous circulation into the animal body, and continue this cycle of blood circulation over relatively long periods of time.

The liquid on the outside of the collodion tubes may be a physiological salt solution or distilled water. If it is water, there is, of course, a rapid loss of the inorganic constituents of the blood. As we shall see later in a discussion of osmotic phenomena, a crystalloid tends to collect in equal concentrations both within and without a membrane to which it is permeable. Accordingly the crystalloidal constituents of the blood pass through the collodion membrane into the outer liquid

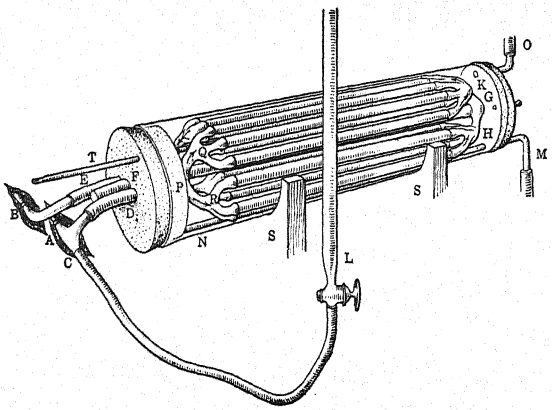


FIG. 22.—Abel's vividiffusion apparatus. Vein at *B*, artery at *C*, anticoagulant added at *L*.

which can be drawn off at intervals and replaced with fresh liquid. In this way the crystalloidal constituents can be separated from the colloidal constituents of the blood.

Abel and his co-workers constructed various vividiffusion cells, the number of collodion tubes ranging from 2 to 32 or more. In some instances vividiffusion was kept up continuously for a period in excess of 10 hours. In preliminary experiments 1 gram of sodium salicylate was slowly injected into the femoral vein of a dog weighing 7 kg. Of the total amount of salicylic acid injected, 19.1 per cent was recovered in the diffusion liquid of the vividiffusion apparatus, whereas only 17.5 per cent was eliminated in the urine during the same period of time, thus showing that the apparatus can compete with the kidneys on favorable terms, at least during a part of the dialysis period.

This apparatus gave definite proof that amino acids circulated in the blood stream in the free state. Abel demonstrated that there was a marked increase in amino acids in the diffusion liquid following the

feeding of a meat diet to a dog which was being used for the vividiffusion experiment. This was apparently the first demonstration that proteins on digestion passed into the blood in the form of free amino acids and were carried in this form from the blood to the various cells and tissues of the body.

In a later paper the authors [110] report on the chemical composition of the material which had diffused through the collodion membranes. In a series of vividiffusion experiments, covering a total of 112 hours, nitrogenous compounds, containing a total of 20 grams of nitrogen, were obtained. A large part of this nitrogen was, as would be expected, urea nitrogen. However, histidine, isobutylhydantoin, alanine, valine, lactic acid, β-oxybutyric acid, and creatinine were identified. Other substances, especially glucose, dialyzed in large quantities. The quantities of the materials which were identified accounted for only a fraction of the total solids which were obtained. Abel notes that the dialysates contained considerable quantities of materials which did not yield to ready identification.

Abel regards the vividiffusion apparatus as similar to an artificial kidney and has suggested that it might be used in case of severe poisoning in order to remove the poisonous elements from the blood stream. Its value in such connection would, of course, depend upon the length of time which had elapsed between the taking of the poison and the beginning of the vividiffusion process. This apparatus should be a valuable tool in physiological studies. Abel notes that with proper technic the blood passing through an individual organ can be passed through a vividiffusion apparatus. In this way the diffusible constituents which any individual organ contributes to the blood stream could be studied.

ELECTRODIALYSIS.—Dialysis which depends only upon diffusion is often an extremely slow process, and it is sometimes impossible to remove the last traces of adsorbed electrolytes. The removal of electrolytes from colloid sols and gels may be hastened and the colloidal material obtained in a much higher degree of purity by dialyzing with the aid of an electric current. Electrolytes in aqueous solutions are dissociated into ions, and these ions move under the influence of a direct current to the anode and the cathode. If the colloidal sol is restrained by suitable membranes, from moving to the anode and cathode compartments, electrolytes can be then almost completely removed. Sheppard, Sweet, and Benedict [111] used this method to prepare ash-free

[110] Abel, J. J., Rowntree, L. G., and Turner, B. B., On the Removal of Diffusible Substances from the Circulating Blood of Living Animals by Dialysis II. Some Constituents of the Blood, *J. Pharmacol.*, 5: 611–623 (1914).

[111] Sheppard, S. E., Sweet, S. S., and Benedict, A. J., Elasticity of Purified Gelatin Jellies as a Function of Hydrogen-ion Concentration, *J. Am. Chem. Soc.*, 44: 1857–1866 (1922).

gelatin. Electrodialysis was carried out in a large wooden cell, in the middle of which was placed a porous inner compartment of silica which extended to and was imperviously cemented to both the bottom and the sides of the wooden cell. The silica container was filled with 5 per cent gelatin, the two end compartments of the wooden cell were filled with distilled water, and the whole electrodialyzed, using 110 volts, direct current, and a gold anode and a silver cathode. By this procedure gelatin containing from 0.02 to 0.03 per cent ash was obtained.

Knaggs, Manning, and Schryver[112] electrodialyzed gelatin by allowing a 10 to 20 per cent gelatin gel to set in the lower part of a bell jar, suspending this in water over a mercury cathode and inserting a platinum anode in water above the gelatin gel. The electrolysis was conducted with 100 to 220 volts, direct current, the water being changed periodically, and the process being continued until there was no further appearance of alkali at the cathode or acid at the anode. The ash content in this way was reduced to 0.02 per cent or less.

Hoffman and Gortner[113] used electrodialysis to study the composition of agar. They showed that the sulfur content of agar is organically bound in the molecule in the form of a substituted sulfuric acid, whereas the agar was freed from other inorganic elements. As usually obtained, agar is neutral in reaction. Electrodialyzed agar, on the other hand, is a strong acid, a 1 per cent solution having a *p*H of approximately 2.5. The silica which was present in the original agar did not pass through the membrane, indicating that it probably was in colloidal form. Thomas and Murray[114] electrodialyzed gum acacia, and prepared an arabic acid, a 1 per cent solution of which had a *p*H value of 2.70. They found, however, that arabic acid was not a sulfuric acid ester, as is agar acid.

Following these studies electrodialysis has come into almost universal use as a method through which colloidal systems can be readily and rapidly freed from electrolyte contamination. Pauli[115] was the first worker to use the technic extensively in the purification of proteins, in which field it is now a routine procedure. It has been applied extensively to the study of soil colloids and to the purification of cellulose, and in general it offers the best method for the purification of lyophilic colloids. Since electrolytes are frequently adsorbed on the

[112] Knaggs, J., Manning, A. B., and Schryver, S. B., Investigations on Gelatin, Part II. Researches on the Methods of Purifying Gelatin, *Biochem. J.*, 17: 473–487 (1923).

[113] Hoffman, W. F., and Gortner, R. A., The Electrodialysis of Agar. A Method for the Preparation of the Free Agar-Acid, *J. Biol. Chem.*, 65: 371–379 (1925).

[114] Thomas, A. W., and Murray, H. A., A Physico-Chemical Study of Gum Arabic, *J. Phys. Chem.*, 32: 676–697 (1928).

[115] Pauli, Wo., and Valko, E., Kolloidchemie der Eiweisskörper, 353 pp., Theodor Steinkopff, Dresden and Leipzig (1933).

surface of the colloid micelles and are held there by electrical forces, the electrolytes become to all intents and purposes a part of the colloidal micelle. It is only by means of an impressed electrical potential that the ions can be removed from the surfaces of the colloidal particles. Probably there is a characteristic "decomposition voltage" which must be impressed upon any particular system in order completely to remove the electrolytes from that system. Accordingly electrolytes can be removed by electrodialysis which cannot be removed by ordinary dialysis or by ultrafiltration.

ELECTRO-ULTRAFILTRATION.—Bechhold and Rosenberg[116] suggested a direct electric current as an aid to ultrafiltration and applied this technic to the purification of gelatin. Figure 23 illustrates diagrammatically their apparatus. On the bottom of a Büchner funnel is placed a perforated metallic disc which forms the cathode. On top of this disc is placed the membrane of the ultrafilter. The liquid to be filtered is then placed above the ultrafilter in the Büchner funnel, and the anode is inserted in liquid in a collodion bag which is suspended in the colloid sol in the Büchner funnel. The anions, present as contaminations in the sol, migrate through the collodion bag to the anode and can be removed by drawing off the liquid in the collodion bag at frequent intervals. The cations pass through the ultrafilter and are washed away in the water which passes through the ultrafilter. An additional phenomenon (electroendosmosis, *vide infra*) hastens the ultrafiltration. Inasmuch as the ultrafilters are generally negatively charged, water is drawn by the electric current through the ultrafilter to the pole having the same sign as the electrical charge on the ultrafilter. Bechhold and Rosenberg found that using this apparatus they were able to ultrafilter colloid sols very rapidly at either atmospheric pressure or slightly reduced pressures, and that electro-ultrafiltration is a much more rapid process than the usual ultrafiltration methods, and that colloidal sols of much higher purity can be obtained. At the same time the phenomenon of electroendosmosis affords a means by which the colloidal sol can be greatly concentrated. Using this technic, they report the separation of gelatin into two protein fractions.

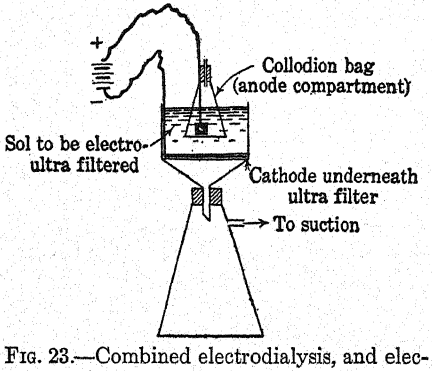


FIG. 23.—Combined electrodialysis, and electro-ultrafiltration. (After Bechhold.)

[116] Bechhold, H., and Rosenberg, A., Elektro-Ultrafiltration von Gelatine und Leim, *Biochem. Z.*, 157: 85–97 (1925).

Czarnetzky [117] reports an electro-ultrafiltration technic of protein sols with the protein kept on the alkaline side of the isoelectric point. In this way the protein micelles are negatively charged. Electro-endosmosis causes a flow of liquid through the membrane (ultrafiltration), whereas cataphoresis of the protein micelles prevents a clogging of the pores of the membrane by the protein. With this technic, sols which resist ultrafiltration by a pressure ultrafilter can be readily ultrafiltered.

Optical Properties of Colloid Systems.—Everyone has observed motes of dust floating in the air when illuminated by a beam of sunlight or in the rays of a projection lantern. The visibility of the dust particles is due to the fact that they act more or less as mirrors reflecting the light rays at an angle so that the observer sees in reality the light source rather than the actual surface of the dust particle. Tyndall [118] was the first to investigate this phenomenon. The light which impinges on a particle is not polarized, whereas the light reflected from the particle is generally strongly polarized. Faraday [119] in exhibiting his red gold sols noted that, "when a light is looked at through the fluid, the latter appears transparent; but when the eye is on the illuminated side, then the fluid is seen opalescent. If a cone of sun's rays be thrown by a lens into the fluid, the illumination of the particles within the cone shows their presence as undissolved bodies." This phenomenon has become known as the *Tyndall phenomenon* and the illuminated path of suspended particles as a *Tyndall cone.*

Colloid systems differ greatly in the intensity with which they show the Tyndall cone. Lyophobic colloids, as a rule, show intense Tyndall cones. Lyophilic colloids, on the other hand, may either show no Tyndall cone or only a faint Tyndall cone. The degree to which a Tyndall cone is shown depends upon the difference between the index of refraction of the dispersions medium and of the disperse phase. If there is a large difference in index of refraction between the two phases, a strong Tyndall cone will result. If there is little or no difference in index of refraction, a faint or no Tyndall cone will be observed. Accordingly, one cannot be certain from the appearance of the Tyndall cone whether one is dealing with a lyophilic or a lyophobic sol. Glass would form a lyophobic sol both in water and in ethyl iodide. The hydrosol would provide a considerable difference in refractive index between the phases and a strong Tyndall cone would result. On the

[117] Czarnetzky, C. J., Electro-Ultra-filtration Apparatus, *Science*, 82: 625–626 (1935).

[118] Tyndall, J., On the Blue Colour of the Sky, the Polarization of Skylight, and on the Polarization of Light by Cloudy Matter Generally, *Phil. Mag.*, (4) 37: 384–394 (1869).

[119] Faraday, M., On the Relations of Gold and Other Metals to Light, *Proc. Roy. Soc.* (*London*), 8: 356–361 (1857).

other hand, the index of refraction between the phases in the organosol would be very slight and only a faint Tyndall cone should result. It should be possible to secure samples of glass having the same refractive index as the ethyl iodide, in which case the lyophobic sol would be optically clear.

Lyophilic sols range from those which show a weak Tyndall cone to those which are practically optically clear. The reason for their transparency lies in the fact that they are highly solvated. A part of the dispersions medium is associated with the disperse phase; the disperse phase is greatly swollen by the dispersions medium which has dissolved in it, and accordingly its index of refraction has been brought very close to the index of refraction of the pure dispersions medium. There is therefore very little light refraction from the surface of the particles.

Live fresh-water medusae may contain as much as 98 per cent of water. When such an organism is floating in the water, it is in many instances practically invisible, because the rays of light pass nearly equally well through the water and through the organism. When it is removed from the water, it is, of course, plainly visible, owing to the difference in refractive index between the air and the organism.

As already noted, Tolman and Vliet[120] devised a Tyndallmeter for the examination of disperse systems. A cloud of colloidal particles or a fine precipitate which is invisible in the ordinary room may be easily visible when a powerful beam of light is focused on the system. Thus, in titrating a potassium cyanide solution with silver nitrate, it is possible by conducting the titration in a strong beam of light to detect the end point where the precipitate of silver cyanide begins to form, some time before it is visible in the absence of the beam. Accordingly a much sharper and much more accurate titration can be made by watching for the first appearance of the Tyndall cone in the solution.

The principle involved in the Tyndall cone is used in analytical chemistry under the name of nephelometry. Kober[121,122] and Kober and Graves[123] have adequately discussed the principles involved. Nephelometric analysis depends upon the refraction of light from the surface of a precipitate and is used where the precipitate is too small in amount for accurate gravimetric determination and where it pos-

[120] Tolman, R. C., and Vliet, E. B., A Tyndallmeter for the Examination of Disperse Systems, *J. Am. Chem. Soc.*, 41: 297–300 (1919).

[121] Kober, P. A., An Improved Nephelometer-Colorimeter, *J. Biol. Chem.*, 29: 155–168 (1917).

[122] Kober, P. A., Technical Applications of Nephelometry, *J. Ind. Eng. Chem.*, 10: 556–563 (1918).

[123] Kober, P. A., and Graves, S. S., Nephelometry (Photometric Analysis) I. History of Method and Development of Instruments, *J. Ind. Eng. Chem.*, 7: 843–847 (1915).

sesses no color, so that colorimetric methods are not applicable. In the determination of atomic weights, where either silver or chlorine is the reference standard, the amount of silver chloride remaining in the wash water and mother liquors is usually accounted for by nephelometric analysis.

When light is reflected from a surface, the short, rapid light rays are usually bent more than are the long, slow rays. Accordingly, the blue, violet, and ultraviolet rays are bent more than the red and yellow rays, and a partial separation of the spectrum results. This is known as opalescence. A sol containing suspended colorless particles may appear to be pale blue. Various-sized particles of the same material may affect the distribution of the reflected rays in different manners. Thus, colloidal gold sols may be colored orange, red, lavender, violet, indigo blue, or black, depending upon the size of the particles of gold in the hydrosol, the smaller particles being at the orange end of the list, the larger ones appearing deep blue or black. Herzfeld and Klinger [124,125] have suggested that in all probability the colors produced when iodine is added to starch or dextrin sols are in reality only manifestations of the size of the colloidal micelles and the different colors do not necessarily indicate chemical differences.

The human eye is sensitive to only a narrow band of radiant energy. Accordingly, the Tyndall cones which we actually see are derived from only that portion of the light to which the retina is sensitive. When ultraviolet light is used as a source of illumination we are unable in many instances to see the Tyndall cone which results. Many systems which are optically empty to the eye may show a marked Tyndall cone when photographed under ultraviolet light. Certain lyophilic systems, which are optically empty with ordinary light, show markedly visible Tyndall cones when a beam of ultraviolet light is projected into them. This phenomenon is known as *fluorescence*, the ultraviolet light being transformed into visible wave lengths. Protein sols and gels in particular show such fluorescence. Svedberg and Tiselius [126] used the fluorescence of proteins in order to render visible the boundary between an egg-albumin sol and the surface of a solution which does not contain protein.

Since a photographic plate may show the presence of a Tyndall cone in molecularly disperse systems, it is obvious that if shorter wave lengths were employed, light refraction would occur from the surface of individual molecules or ions. This is actually the case. X-rays are

[124] Herzfeld, E., and Klinger, R., Zur Chemie der Polysaccharide, *Biochem. Z.*, 107: 268–294 (1920).

[125] Herzfeld, E., and Klinger, R., Berichtung und Ergänzung zu unserer Arbeit: "Zur Chemie der Polysaccharide," *Biochem. Z.*, 112: 55–60 (1920).

[126] Svedberg, The, and Tiselius, A., A New Method for Determination of the Mobility of Proteins, *J. Am. Chem. Soc.*, 48; 2272–2278 (1926).

refracted from the surface of individual atoms within crystals so as to give the familiar spatial orientation of atoms from which so much of our recent knowledge of crystal structure has been derived.

The following diagram illustrates certain of the optical properties of systems having various degrees of dispersion:

Atoms	Molecularly Dispersed Systems	Colloidal Systems	Suspensions
	Amicrons	Ultramicrons	Microns
Tyndall phenomenon by Röentgen rays	Tyndall phenomenon by ultraviolet rays	Tyndall phenomenon by visible light rays	Turbid appearance / Coarse particles visible

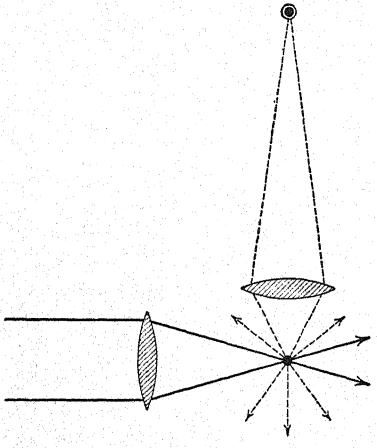


FIG. 24.—The diffraction of light impinging upon an ultramicroscopic particle, the axes of the illuminating and diffracted rays being at right angles to each other. (Solid lines, illuminating rays; dotted lines, diffracted rays.)

Although considerable qualitative information in regard to colloid systems can be obtained by use of the Tyndall beam, it is not directly applicable to a careful study of colloid systems. However, Siedentopf and Zsigmondy [127] devised an instrument known as the ultramicroscope, utilizing the general principles of the Tyndall phenomenon. If a powerful beam of light is focused to a point within a colloid sol and if the rays are refracted from the surface of the disperse phase, one may view the position and the Brownian movement of individual particles through a microscope. Figure 24 shows diagrammatically the path of such light rays from a light source to a colloid particle through a lens to the observer's eye.

Figure 25 shows the arrangement of the Siedentopf-Zsigmondy slit ultramicroscope for the examination of ultramicroscopic particles. The principle is the same as the technic sometimes used in signaling in warfare and boy scout activities, and known as heliography. One person on a hilltop, perhaps several miles away, holds in his hand a small mirror, about two or three inches in diameter, in such a way as to catch the sun's rays; the rays are flashed in the Morse code across the intervening valley to the receiver stationed at a distance. The re-

[127] Siedentopf, H., and Zsigmondy, R., Über Sichtbarmachung und Grössenbestimmung ultramikroskopischer Teilchen, mit besonderer Anwendung auf Goldrubingläser, *Ann. Physik* (4) 10: 1–39 (1903).

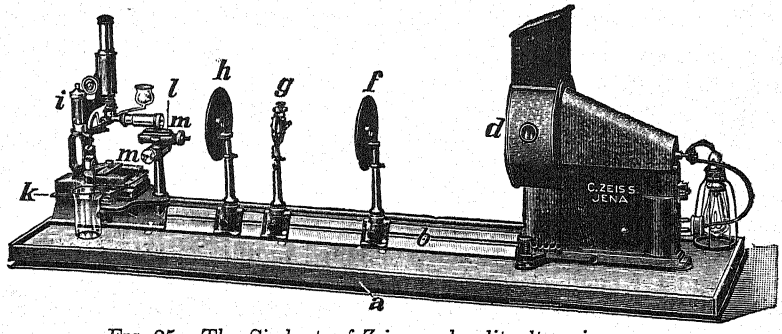


FIG. 25.—The Siedentopf-Zsigmondy slit ultramicroscope.

ceiver does not observe the mirror, which itself is far too small to be seen with the unaided eye across the intervening distance, but rather he sees the light source, the sun, reflected from the surface of the mirror. In the same way the observer, looking into an ultramicroscope, does not see the colloid particle. The particle is too small to be seen with the magnification employed, so that the individual bright particles which are viewed in the ultramicroscope do not indicate either the form or the size of the ultramicroscopic particle but instead they merely act as mirrors, and the observer sees reflected from the surface of such a mirror the source of light which is used for illumination, perhaps a carbon arc or for more intense illumination the direct image of the sun.

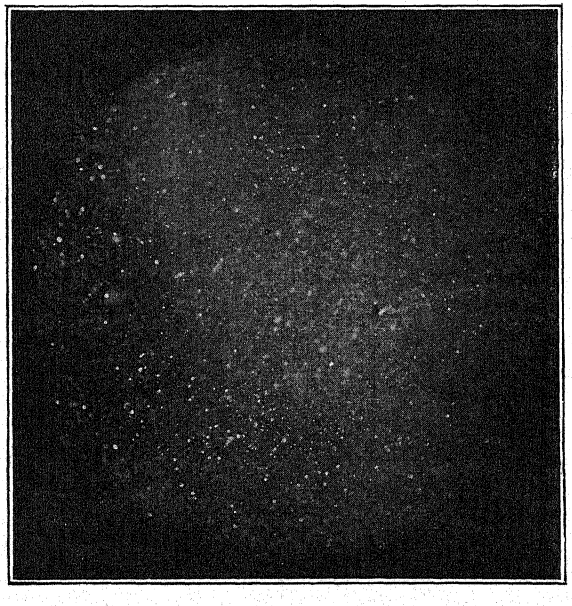


FIG. 26.—Photomicrograph of the gold particles in a red gold sol as viewed with a Siedentopf-Zsigmondy slit ultramicroscope.

Figure 26 is a photomicrograph of a Zsigmondy red gold sol taken as viewed through a Siedentopf-Zsigmondy slit ultramicroscope. The

system which was photographed contained only 0.012 mg. of gold in 500 cc. volume. Nevertheless this 0.012 mg. of gold was sufficient to form approximately 625,000,000,000 colloid particles.

The ultramicroscopic field which is shown in Fig. 26 is still far too concentrated for quantitative studies using the ultramicroscope, but it illustrates the point which it is desired to emphasize, *i.e.*, the immense number of colloid particles that may result from a relatively small weight of material.

A gold hydrosol is particularly adapted to ultramicroscopic studies because of the completeness with which light is reflected from a gold surface. If an egg-albumin sol or the hypothetical glass-ethyl iodide sol already noted were examined under the ultramicroscope, the field would be practically empty. Accordingly the test of ultramicroscopic visibility is not necessarily a valid criterion as to whether a given disperse phase is lyophilic or lyophobic, and the absence of ultramicrons in the ultramicroscope is no proof that truly colloidal micelles are not present.

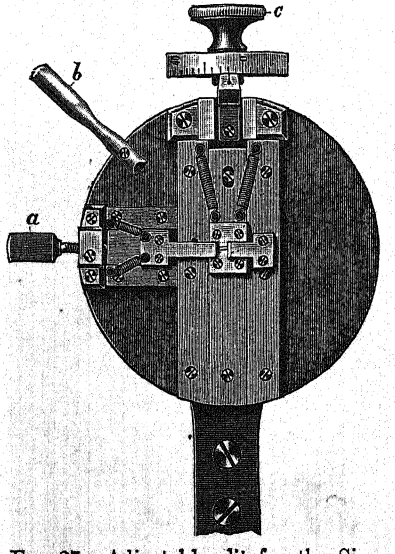


FIG. 27.—Adjustable slit for the Siedentopf-Zsigmondy ultramicroscope.

Referring again to Fig. 25, the beam from the electric arc in the lamp-housing *d*, passes through the lens, *f*, which concentrates the beam and throws it on an adjustable slit, *g*. The arrangement of this adjustable slit is shown in Fig. 27. The slit can be adjusted by the micrometer screws *a* and *c*, the latter having attached to it a graduated drum reading in microns. Accordingly, the width of the slit in one direction can be accurately known. Lever *b*, Fig. 27, affords a means by which the adjustable slit may be rotated at right angles, so that the slit of known dimensions can be altered from depth to breadth in the solution. In this way an area of known dimensions can be illuminated, the number of particles in this known area can be counted, and accordingly the concentration of ultramicroscopic particles per cubic centimeter can be ascertained. As the beam of light passes through slit *g*, it again passes through a second lens, *h*, and into a microscope objective, *l*, from which it emerges and passes through a quartz window of the cell containing the colloid sol under investigation.

The cell which is used is illustrated in Fig. 28, the beam of light

passing through quartz window, c_2, the microscope through which the reflected beam passes to the observer's eye being focused through the quartz window, c_1. Figure 29 shows diagrammatically the appearance of the beam of light passing through window c_2, into the colloid sol. The micrometer screws, l and m, Fig. 25, adjust the focus of the objective, AA, Fig. 29, until the beam of light comes to a focus in the center of the visible field, as shown in Fig. 29.

Burton [128] gives an excellent discussion of the precautions that must be observed in order to make studies with the ultramicroscope. Similarly, the precautions noted by Zsigmondy [129] should be taken carefully

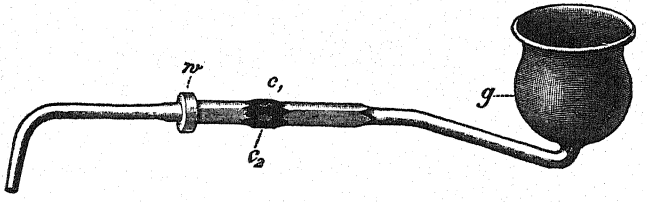


FIG. 28.—Cell with quartz windows for the Siedentopf-Zsigmondy slit ultramicroscope.

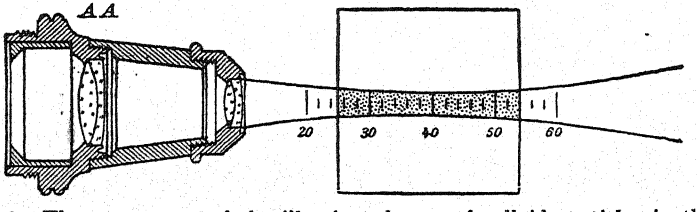


FIG. 29.—The appearance of the illuminated area of colloid particles in the cell of the Siedentopf-Zsigmondy slit ultramicroscope.

into consideration, provided that the instrument is to be used as a research tool. The photomicrograph reproduced in Fig. 18 was secured by making use of an ultramicroscope.

Other forms of the ultramicroscope which are extremely useful in biological investigations are those known as the paraboloid condenser or the still more improved type, the cardioid condenser, as developed by Siedentopf.[130,131] Both the paraboloid condenser and the cardioid con-

[128] Burton, E. F., The Physical Properties of Colloidal Solutions, Second Edition, Chap. III, Longmans, Green and Company, New York (1921).

[129] Zsigmondy, R., Colloids and the Ultramicroscope, translated by Jerome Alexander, John Wiley & Sons (1909).

[130] Siedentopf, H., Über ultramikroskopische Abbildung, *Z. wiss Mikroskop.*, 26: 391–410 (1909), und Über einen neuen Fortschritt in der Ultramikroskopie, *Ber. deut. physik. Ges.*, 8: 6–47 (1910).

[131] Siedentopf, H., Über bisphärische Spiegelkondensoren für Ultramikroskopie, *Ann. Physik* (4) 39: 1175–1184 (1912).

denser are designed so as to fit directly in the frame of the ordinary high-grade microscope replacing the ordinary substage condenser. Figure 30 shows diagrammatically the path of the light rays through a paraboloid condenser. The shaded area, D, is an optical glass block, the curved faces of which are highly silvered so as to form reflecting mirrors. A black glass disc is inserted over a portion of the lower surface so as to provide a black background directly under the objective of the microscope. The light rays, A and A', reflected from the mirror of the microscope, pass through the clear portion of the optical-glass block and are reflected from the mirror surfaces at such an angle that they focus at a point slightly below the plane of contact of the glass slide, E, with the cover glass, F. The slide, E, contains a slight depression in which the colloidal system under investigation is placed, the light rays coming to focus within the colloid system lying in this depression. The microscope objective is then focused on the point at which the light rays converge within this depression.

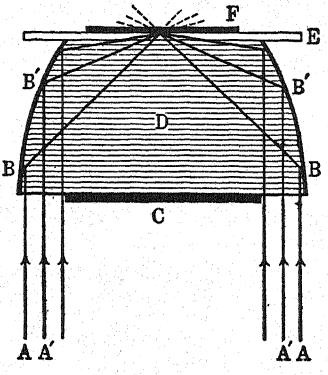


FIG. 30.—A diagrammatic representation of the path of the light rays in a paraboloid condenser.

Figure 31 shows the more elaborate and more nearly optically perfect arrangement of lenses in the cardioid condenser. The light rays in this instance are focusing in a block of uranium glass which shows strong fluorescence. One precaution which must always be observed when either the paraboloid condenser or the cardioid condenser is used is to make contact of the microscope slide with the surface of the condenser through the medium of an immersion oil. If the oil is not used, the rays will not pass from the surface of the condenser into and through the glass slide, owing to refraction at a glass-air interface.

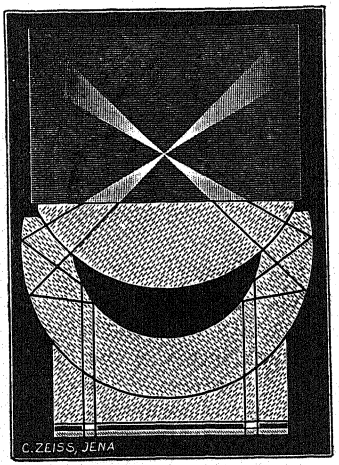


FIG. 31.—The path of the light rays in a cardioid condenser. A uranium glass plate has been placed above the condenser to show the focus of the rays which have passed through the condenser. Where these rays converge is the point upon which the microscope should be focused for the study of colloid systems.

Another instrument which appears to have possibilities in the field of ultramicroscopy is a Spierer lens used with a Spierer condenser. Seifriz [132] applied this lens system to the ultramicroscopy of protoplasm, and Thiessen [133] applied it to studies of the cell-wall structure of various woods and to coals and found that by its use he could distinguish evidence of cellular structure even in high-grade bituminous coals. Other workers have claimed that the Spierer lens gives optical diffraction patterns which are artificial and do not indicate structural elements. However, the author has found the Spierer lens to be a useful tool and to reveal characteristic patterns in cellular materials which cannot be differentiated by other optical technics.

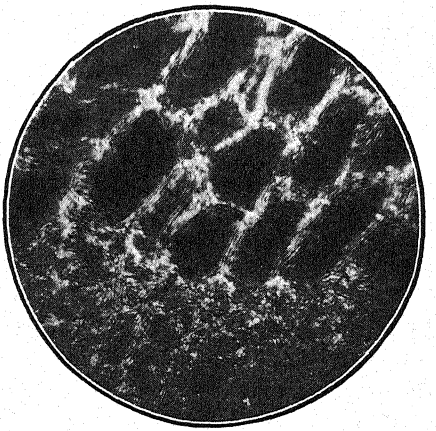


FIG. 32.—The structure of partially decayed coniferous wood as revealed by the Spierer lens. Note micellae in cell walls. (Photograph, courtesy of Dr. Reinhardt Thiessen.)

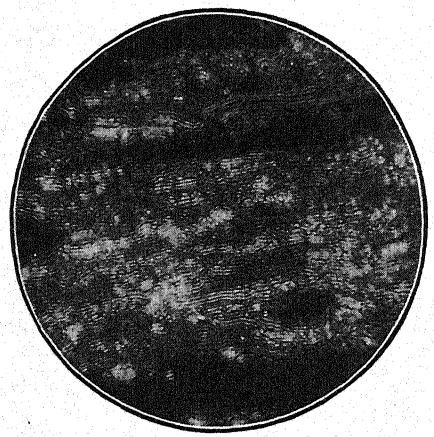


FIG. 33.—The structure of bituminous coal as revealed by the Spierer lens. Note micellae in old cell walls. (Photograph, courtesy of Dr. Reinhardt Thiessen.)

The characteristic of the Spierer lens is a tiny gold mirror plated on one of the lenses of the microscope. The condenser is a dark-field condenser with a tiny core of optical glass directly through the center of the condenser. Light from the microscope mirror passes through this optical-glass core, focuses on the tiny gold mirror, and is reflected at an angle from the gold mirror to the *surface* of the material under observation on the microscope slide and is again refracted from this material back into the

[132] Seifriz, W., The Spierer Lens and What It Reveals in Cellulose and Protoplasm, *J. Phys. Chem.*, 35: 118–129 (1931).

[133] Thiessen, R., Physical Structure of Coal, Cellulose Fiber, and Wood as Shown by Spierer Lens, *Ind. Eng. Chem.*, 24: 1032–1041 (1932).

microscope lens and to the eye of the observer. At the same time the core of optical glass in the condenser acts like the paraboloid condenser and transmits light which strikes the object on the microscope slide from below at an angle, giving an ultramicroscopic field. The object accordingly is illuminated with light transmitted through the object, with light striking the object at an angle so that the light is refracted from the object, and with light reflected on the object from above, and all these three sources of light are combined in the eyepiece of the microscope. Figure 32, taken from the paper of Thiessen, shows the appearance of the cell walls in a coniferous wood in which decay is well advanced, and Fig. 33, also from his paper, shows the appearance of a thin section of bituminous coal, both as viewed under the Spierer lens.

The limit of the size of the particle which becomes discernible in the ultramicroscope depends upon two factors, the intensity of illumination and the difference in refractive index existing between the disperse phase and the dispersions medium.

Using direct sunlight and extreme precautions, Zsigmondy was able to differentiate particles of colloidal gold as small is 1.7 mμ from the water in which they were dispersed. As Siedentopf [134] points out, if molecules could be got far enough apart and if strong enough illumination were possible, molecules themselves could be differentiated in the ultramicroscope. However, sunlight is far too dim a light source, and since sunlight is the most intense source of light which we have available, the theoretical possibilities of the ultramicroscope cannot be attained. The usual lower limit of resolution of the Siedentopf-Zsigmondy ultramicroscope lies at about 5 mμ under ordinary conditions of illumination, with a somewhat higher value for the cardioid form of the ultramicroscope and a still higher value for the paraboloid condenser.

As noted above, the slit ultramicroscope can be utilized to determine approximately the size of the colloid particle on the assumption that the particles in question are spheres and that the specific gravity of the colloidal micelle is identical with the specific gravity of the same material in mass. Using the rotating slit, a definite volume of a dilute sol can be illuminated, and the number of points of light, each indicating the position of a particle in this volume, can be counted. The weight of the disperse phase in the original sol can be ascertained by evaporating a portion of the sol to dryness and determining its solid content.

Let us assume that a silver sol was prepared which contained 6.8 mg. of silver in 100 cc. volume. The original sol was diluted 100 times, and when viewed in the ultramicroscope was found to contain

[134] Siedentopf, H., On the Rendering Visible of Ultra-Microscopic Particles and of Ultra-Microscopic Bacteria, *J. Roy. Microscop. Soc.*, 573–582 (1903).

300 particles per 0.1 cu. mm. The original sol, therefore, contained 30,000,000,000 particles, weighing a total of 6.8 mg. The mean volume of the particles in the sol was 2.2×10^{-14} cc., assuming a specific gravity of 10.5. If the particles are spheres with an average radius, r, we have

$$\tfrac{4}{3}\pi r^3 = 22 \times 10^{-15} \tag{23}$$

Accordingly $r = 1.7 \times 10^{-5}$ cm. or 0.17 μ. The silver particles, therefore, lie at the extreme upper limit of the colloid realm in so far as their size is concerned.

The errors in such a measurement are: (1) the difficulty of accurately counting the number of particles, owing to their incessant Brownian movement; (2) the fact that the particles may not be of uniform size (some may be microns, some ultramicrons, some amicrons, and a part of the material in the disperse phase may be even molecularly dispersed); and (3) the fact that the size of the particle may be affected by dilution.

A second method depends upon a count of the number of particles in a definite volume, comparing this with the total mass of material in a given amount of the sol, and a measurement of the distance between the particles as viewed in the ultramicroscope. The same errors apply to this method. The equation which is utilized here is

$$r = \sqrt[3]{\frac{M}{d^x}} \tag{24}$$

where M = the weight of disperse phase in a unit volume of the sol;
d = density of the material making up the disperse phase;
x = the average distance in centimeters between the particles comprising the disperse phase.

The protoplasm of a *Spirogyra* cell viewed in an ultramicroscope is found to be full of light points in regions which are optically void when viewed by ordinary microscopic technic. The ultramicroscope, particularly in the form of the paraboloid or cardioid condenser, should be more generally applied to biological problems.

CHAPTER IV

HYDROGEN-ION CONCENTRATION

In order to discuss adequately certain properties of colloid systems and to understand certain biological reactions, it is necessary to diverge at this point and consider briefly the subject of hydrogen-ion concentration.

It is impossible within the space at our disposal to cover the details regarding hydrogen-ion concentration, including the theoretical background and all the technic involved in the various methods used for measurement. We are particularly fortunate in having available such excellent treatises as those of Clark [1] and Michaelis,[2, 3] which are indispensable adjuncts to the library of anyone working in this field.

Water is in many respects a unique substance. It is liquid at ordinary temperatures, whereas compounds more or less similar in structure, such as H_2S, SO_2, NH_3, etc., are gaseous; in the liquid state it has a very high surface tension differentiating it from other liquids, it has a minimum volume at + 4° C., expanding on solidifying; and, last but not least, solutions of many substances in water as a solvent possess the ability to conduct an electric current. If acids or bases or salts are dissolved in water, the solution becomes a conductor for an electric current. Accordingly, such substances are known as electrolytes.

Arrhenius, in 1887, postulated that when electrolytes were dissolved in water they were dissociated into their corresponding ions and that it was these charged ions through which the flow of electric current took place. The separation of an electrolyte into its component ions is known as *dissociation.*

Arrhenius noted that electrolytic conductance in solution was not strictly proportional to the amount of electrolyte which was dissolved. He accordingly suggested that at infinite dilution complete dissociation took place, whereas in more concentrated solutions the dissocia-

[1] Clark, W. Mansfield, The Determination of Hydrogen Ions, Third Edition, Williams and Wilkins Company, Baltimore (1928).

[2] Michaelis, L., Hydrogen Ion Concentration, Vol. I. Principles of the Theory, translated by W. A. Perlzweig, Williams and Wilkins Company, Baltimore (1926).

[3] Michaelis, L., Die Wasserstoffionenkonzentration, Julius Springer, Berlin (1914).

tion was not entirely complete, part of the original solute remaining in solution in an undissociated condition. In accordance with this view, if we dissolve sodium chloride in water, the following equilibrium would be set up:

$$NaCl \rightleftharpoons Na^+ \text{ and } Cl^-$$

In a dilute solution the equilibrium would be shifted toward the right until at infinite dilution all of the sodium chloride was dissociated. In a concentrated solution the equilibrium would be shifted more and more toward the left.

The views of Arrhenius in regard to the dissociation of electrolytes have been altered somewhat by the recent researches of physicists and physical chemists, which have thrown doubt on the existence of individual molecules, such as is represented by NaCl. The X-ray crystal structure of sodium chloride does not indicate the presence of a definite molecule of NaCl, but rather of atoms of sodium and chlorine spaced at equal distances from each other throughout the crystal structure. Accordingly, it has been suggested that an electrolyte such as sodium chloride is completely dissociated even in the solid state. If this be so, it must be obvious that it would also be completely dissociated in water. It would, therefore, be theoretically impossible to have a solution where the sodium chloride was 98 per cent dissociated. Solutions of sodium chloride can, however, be obtained which have only 98 per cent of the electrolytic conductance theoretically possible. Therefore, the term, *activity*, has largely displaced the term dissociation, when solutions of strong electrolytes are under consideration. On the assumption that dilute solutions of strong electrolytes are completely dissociated but that as the concentration increases, the anions are more or less decreased in their activity by the adjacent cations, and correspondingly the activity of the cations may be altered by the adjacent anions, a theory has been built up substituting changes in activity for the older conception of changes in dissociation. The theory of complete dissociation which has proved so valuable with strong electrolytes still fails to explain the behavior of solutions of weak electrolytes, such as acetic acid or ammonia, where the theory of incomplete dissociation must still be retained.

The above discussion is inserted at this point merely to indicate some of the recent trends. It is probably more strictly correct to speak of *hydrogen-ion activity* than of *hydrogen-ion concentration*, and throughout the following discussion it will be well to bear in mind that the term hydrogen-ion concentration refers to the "apparent" hydrogen-ion concentration, *i.e.*, the hydrogen-ion activity, rather than to the actual normality of ionized hydrogen present in the solution, for the methods which are employed for the measurement of hydrogen-

ion concentration in reality measure the so-called hydrogen-ion activity.[4]

Conventionally we write the hydrogen ion by the symbol H^+, and in the remainder of this book we will continue that symbolism. It must be emphasized, however, that this is only a symbol and does not represent the actual state of affairs, for in reality H^+ is a naked proton and as such is almost infinitesimal in size and accordingly is able to penetrate within the structure of the first molecule with which it comes in contact. In an aqueous system this would probably be a water molecule, so that the actual structure might be represented by $(H_3O)^+$, the *hydronium* (or oxonium) *ion*.[5]

Bernal and Fowler[6] point out that the mobilities of the "hydrogen" and hydroxyl ions are 32.5×10^{-4} and 17.8×10^{-4} cm./sec./volt/cm. respectively, and that all the other ions have mobilities in the neighborhood of 6.7×10^{-4} cm./sec./volt/cm. They account for this by suggesting that, if the ion OH_3^+ comes near an H_2O molecule, there is the possibility that the extra proton in the OH_3^+ can jump over to the other molecule. The net effect is as though the molecules had changed positions, but because of the small mass of the proton its transfer occurs at a higher rate than would be possible if the ions had the same mass. When an external electric field is set up for measuring mobility, it causes the protons to "hop" along from molecule to molecule in the direction of the electric field. In this way the body of the liquid acts as a "conductor of protons." A similar argument can be applied to explain the mobility of OH^- which in this case is simply a water molecule which has lost a proton.

Huggins[7, 8] adds to this picture by pointing out that the hydronium ion must be regarded as not only OH_3^+, but that higher polymers, such

[4] For those who wish to go further into the theories underlying the activity of ions, the following books are recommended: Eucken, A., Jette, Eric R., and LaMer, V. K., Fundamentals of Physical Chemistry, McGraw-Hill Book Company, New York (1925); Lewis, G. N., and Randall, M., Thermodynamics and the Free Energy of Chemical Substances, McGraw-Hill Book Company, New York (1923); and Kolthoff, I. M., Acid-Base Indicators, translated by Charles Rosenblum, The Macmillan Company, New York (1937). See also Lewis, G. N., and Randall, M., The Activity Coefficient of Strong Electrolytes, *J. Am. Chem. Soc.*, 43: 1112–1154 (1921), and The Theory of Strong Electrolytes, Faraday Society, London (1927).

[5] Kolthoff, I. M., The Confusion in the Expression of the So-Called "Hydrogen Ion Concentration" of a Solution and a Review of Brönsted's Conception of Acidity and Basicity, *Rec. trav. chim.*, 49: 401–414 (1930).

[6] Bernal, J. D., and Fowler, R. H., A Theory of Water and Ionic Solution, with Particular Reference to Hydrogen and Hydroxyl Ions, *J. Chem. Phys.*, 1: 515–548 (1933).

[7] Huggins, M. L., Hydrogen Bridges in Ice and Liquid Water, *J. Phys. Chem.*, 40: 723–731 (1936).

[8] Huggins, M. L., Hydrogen Bridges in Organic Compounds, *J. Org. Chem.*, 1: 407–456 (1936).

as $O_2H_5^+$, $O_3H_7^+$, ... are equally probable inasmuch as water exists in an indefinite number of polymers of H_2O.

If we have an acid of the type HA, its dissociation into H^+ and A^- is reversible and the dissociation may be expressed as

$$HA \rightleftharpoons H^+ \text{ and } A^-$$

Applying the equation for mass action and expressing concentration by inclosing the quantities in brackets, we have the equation

$$\frac{[H^+] \times [A^-]}{[HA]} = K_a \tag{25}$$

where $K_a =$ the ionization or dissociation constant.

Equation (25) states that for any given acid the product of the concentration of the anion and the cation divided by the concentration of the remaining undissociated acid is a constant. If K_a is large, the greater part of the acid is dissociated into hydrogen ions and anions, whereas if K_a is small, the greater part of the acid is present in the form of undissociated molecules. Accordingly the dissociation constant K_a is a measure of the relative strength of an acid.

In a similar way the dissociation of a base may be represented by

$$\frac{[B^+] \times [OH^-]}{[BOH]} = K_b \tag{26}$$

where $K_b =$ the dissociation constant of a base and bears the same relation in regard to the alkalinity of a solution that K_a has with respect to the acidity.

Inasmuch as water is the common solvent and inasmuch as it dissociates somewhat into hydrogen and hydroxyl ions, one of which is characteristic of the dissociation of acids, the other of bases, the dissociation constant of water becomes a very important quantity in the calculation of hydrogen-ion concentration. The dissociation of water may be represented by

$$\frac{[H^+] \times [OH^-]}{[HOH]} = K_w \tag{27}$$

This equation states that the product of the concentration of hydrogen and hydroxyl ions divided by the concentration of the undissociated water is a constant. It is usually regarded as safe to ignore the denominator in this equation, inasmuch as it appears to be approximately constant. The equation accordingly is usually expressed as

$$[H^+] \times [OH^-] = K_w \tag{28}$$

where $K_w =$ the dissociation constant of water.

From equation (28) we read that, no matter how great is the concentration of hydrogen ions, there must always remain sufficient hydroxyl ions in the solution to satisfy the equation, and conversely no matter how great the concentration of hydroxyl ions, there must always be a residue of hydrogen ions present to satisfy the above equation. The value for the dissociation constant of water is, therefore, a very important constant, and its value is approximately 10^{-14}. It is usually expressed as $\log \frac{1}{K_w}$ which has a value of 13.995 at 22° C.[9] If K_w at 22° C. has a value of 1×10^{-14} and the hydrogen- and hydroxyl-ion concentrations are equal, then pure water at this temperature is 1×10^{-7} (or one-ten-millionth) normal with respect to hydrogen and hydroxyl ions. One gram molecule of any substances contains approximately 6.061×10^{23} molecules. Accordingly in pure water there would be $6.061 \times 10^{23} \times 10^{-7}$ hydrogen ions per liter. One liter of water contains 55.56 gram molecules or $55.56 \times 6.061 \times 10^{23}$ water molecules. This same liter, on the other hand, contains only 6.061×10^{16} hydrogen ions. Accordingly only one molecule of water in every 555,000,000 molecules is dissociated into hydrogen and hydroxyl ions.

From equations (25), (26), and (28) it is possible to present an equation which applies equally well to all solutions of acids and bases. Equation (25) may be rewritten

$$\frac{1}{[H^+]} = \frac{[A^-]}{K_a[HA]} \tag{29}$$

or it may be expressed by using the reciprocal of $[H^+]$ and taking the logarithm of each side of the equation as

$$\log \frac{1}{[H^+]} = \log \frac{1}{K_a} + \log \frac{[A^-]}{[HA]} \tag{30}$$

The logarithm of the reciprocal of the hydrogen-ion concentration is expressed in the term *p*H. This term, which is commonly met with in biological and biochemical studies where hydrogen-ion concentration is referred to, was first introduced by Sörensen[10, 11] for reasons which will be referred to later.

[9] Clark, *loc. cit.*, gives on page 45 a table for the variation of $\log \frac{1}{K_w}$ with temperature, together with the *p*H of the neutral point at the various temperatures.

[10] Sörensen, S. P. L., Enzymstudien. II. Über die Messung und die Bedeutung der Wasserstoffionen-konzentration bei enzymatischen Prozessen, *Biochem. Z.*, 21: 131–304 (1909).

[11] Sörensen, S. P. L., Études enzymatiques. II. Sur la mesure et l'importance de la concentration des ions hydrogène dans les réactions enzymatiques, *Compt. rend. trav. lab. Carlsberg*, 8: 1–168 (1909).

By referring to equation (28) it will be noted that the term pH can be used equally well to express either the degree of acidity or the degree of alkalinity of a solution, inasmuch as that equation could be rewritten

$$\frac{1}{[H^+]} \times \frac{1}{[OH^-]} = \frac{1}{K_w} \tag{31}$$

or

$$p\text{H} + p\text{OH} = pK_w \tag{32}$$

When a substance yielding H^+ or OH^- ions is added to water, the ionization of the water is repressed so that the hydrogen-ion concentration decreases as the hydroxyl-ion concentration increases, and *vice versa.* Therefore *a decrease in hydrogen-ion concentration may be used to express an increase in hydroxyl-ion concentration, thus permitting one scale to be used for the measurement of both acidity and alkalinity.*

The fact that the symbol pH can be used to designate either acidity or alkalinity makes it especially useful in biochemical studies where the degree of acidity or alkalinity ranges around the neutral point. Table XII shows the relationship between pH and the concentration of hydrogen or hydroxyl ions which may be present in solution.

An additional reason for using the symbol pH lies in the fact that we can express, in 14 units, acidities which range from a solution which is 0.1 normal in terms of hydrogen ions to a solution which is one one-hundred trillionth normal (10^{-14}) in terms of hydrogen ions. If we desired to plot the actual hydrogen-ion concentration, C_H, or the hydrogen-ion activity, C_{Ha}, allowing 1 mm. for the unit between 10^{-13} normal and 10^{-14} normal, we would need a piece of paper 111,111 + kilometers or approximately 69,444 miles long in order to include the

TABLE XII

SHOWING THE RELATIONSHIP BETWEEN pH, pOH, AND THE NORMALITY OF A SOLUTION IN TERMS OF ACTIVE HYDROGEN OR HYDROXYL IONS

pH 1	= 0.1 N	Hydrogen Ions and	10^{-13} N	Hydroxyl Ions	= pOH 13
pH 2	= 0.01 N	Hydrogen Ions and	10^{-12} N	Hydroxyl Ions	= pOH 12
pH 3	= 0.001 N	Hydrogen Ions and	10^{-11} N	Hydroxyl Ions	= pOH 11
pH 4	= 0.0001 N	Hydrogen Ions and	10^{-10} N	Hydroxyl Ions	= pOH 10
pH 5	= 10^{-5} N	Hydrogen Ions and	10^{-9} N	Hydroxyl Ions	= pOH 9
pH 6	= 10^{-6} N	Hydrogen Ions and	10^{-8} N	Hydroxyl Ions	= pOH 8
pH 7	= 10^{-7} N	Hydrogen Ions and	10^{-7} N	Hydroxyl Ions	= pOH 7
pH 8	= 10^{-8} N	Hydrogen Ions and	10^{-6} N	Hydroxyl Ions	= pOH 6
pH 9	= 10^{-9} N	Hydrogen Ions and	10^{-5} N	Hydroxyl Ions	= pOH 5
pH 10	= 10^{-10} N	Hydrogen Ions and	0.0001 N	Hydroxyl Ions	= pOH 4
pH 11	= 10^{-11} N	Hydrogen Ions and	0.001 N	Hydroxyl Ions	= pOH 3
pH 12	= 10^{-12} N	Hydrogen Ions and	0.01 N	Hydroxyl Ions	= pOH 2
pH 13	= 10^{-13} N	Hydrogen Ions and	0.1 N	Hydroxyl Ions	= pOH 1
pH 14	= 10^{-14} N	Hydrogen Ions and	1.0 N	Hydroxyl Ions	= pOH 0

whole range of C_H between *p*H 1 and *p*H 14. In terms of *p*H the entire graph could be placed on an ordinary sheet of coordinate paper. *It is essential to remember that the pH scale is a logarithmic scale.* Accordingly *p*H 6 represents a solution containing 10 times the concentration of hydrogen ions that are present in a solution having a *p*H of 7. A solution with a *p*H of 5 is 100 times as acid as a solution with a *p*H of 7. A solution with a *p*H of 4 is 1000 times as acid as a solution with a *p*H of 7. Likewise *the fractions in a pH scale do not represent arithmetical values but rather logarithmic values.* Thus, the difference in acidity between *p*H 5.0 and 5.1 is many times greater than that between *p*H 5.9 and *p*H 6.0.

Expressing acidity or alkalinity in terms of the concentration of the hydrogen or hydroxyl ions or in terms of *p*H permits us to distinguish between the strong acids or bases and the so-called weak acids or bases. Hydrochloric acid and acetic acid ionize respectively as follows:

$$HCl \rightleftharpoons H^+ \text{ and } Cl^-$$

$$HC_2H_3O_2 \rightleftharpoons H^+ \text{ and } C_2H_3O_2^-$$

Hydrochloric acid, however, is a strong acid, and ion concentration measurements indicate that all or practically all of the hydrogen ions in a 0.1 normal solution of hydrochloric acid are active. On the other hand, only a small fraction of the available hydrogen ions in acetic acid are active (less than 1 per cent in an 0.1 normal solution). We accordingly say that only a small fraction of the acetic acid is dissociated, the major portion remaining as undissociated acetic acid molecules.

If we were to titrate deci-normal solutions of acetic and hydrochloric acids with standard sodium hydroxide, using a suitable indicator, we would find that both acids required the same amount of sodium hydroxide for neutralization. The amount of standard sodium hydroxide which would be required represents the *titratable acidity*, but this determination tells us nothing in regard to the *actual acidity* of the solution at any particular period of time. *The titratable acidity is a measure of the potential acidity, i.e., the quantity of hydrogen ions which can be made to combine with a base. Hydrogen-ion-concentration measurement, on the other hand, is a measure of the actual concentration (or activity) of hydrogen ions present in a given system at a given time, and is therefore the only true measure of how such a solution will affect another system which is sensitive to hydrogen or hydroxyl ions.* Hydrochloric acid is a strong acid, and its hydrogen ions in solution approach ideal activity. A normal solution of hydrochloric acid is a poison, whereas a normal solution of acetic acid is a fairly weak artificial vinegar. Both solutions have the same potential (*i.e.*, titratable) acidity, but their hydrogen-ion activities are widely different.

The reactions of the biological organism toward acids and bases are not controlled by changes in potential acidity but rather are affected by changes in the actual activity of hydrogen ions. Table XIII shows

TABLE XIII

THE APPROXIMATE RELATIONSHIP BETWEEN THE NORMALITY OF ACID SOLUTIONS (POTENTIAL ACIDITY) AND THE *p*H (THE NEGATIVE LOG OF THE ACTUAL ACIDITY) AT 25° C.*

Normality of Solution	*p*H of Acid Solution at 25° C.				
	Lactic	Acetic	Ortho-Phosphoric	Hydrochloric	Oxalic
0.500	1.89	2.50	1.33	0.46	1.03
0.200	2.11	2.69	1.59	0.81	1.29
0.100	2.27	2.85	1.79	1.09	1.52
0.040	2.49	3.08	2.05	1.46	1.82
0.020	2.67	3.24	2.26	1.77	2.10
0.010	2.82	3.39	2.49	2.06	2.36
0.005	3.00	3.54	2.73	2.35	2.62
0.002	3.23	3.76	3.08	2.73	3.00

* The values of *p*H are probably not exact from the standpoint that they could be used as physicochemical constants. They represent, however, actual values which were obtained on the solutions in question by using electrometric methods of determining hydrogen-ion concentration. These values should not be used except as representing first approximations and are inserted here largely for the purpose of illustrating the variation which may be obtained in actual acidity when solutions of the same potential acidity are compared.

the relationship between the potential acidity and the actual acidity of solutions of certain of the common acids.

Figures 34 and 35 [12] illustrate the differences which may be observed in biochemical systems which are sensitive to the action of acids. In Fig. 34 the viscosity of a 20 per cent wheat flour-in-water suspension is plotted against the normality of the various acids which were added to the suspension. It will be noted that there is a great difference in the form of the viscosity curves with acid concentration. When, however, the above data were plotted not against the potential acidity of the solution but against the actual acidity, *i.e.*, the hydrogen-ion concentration, Fig. 35 resulted. Instead of a maximum being reached at various points on the curve, as in Fig. 34, the maximum viscosity is in all instances reached at an acidity of approximately *p*H 3.0, and the acids instead of behaving in entirely dissimilar man-

[12] Gortner, R. A., and Sharp, P. F., The Physico-chemical Properties of Strong and Weak Flours. III. Viscosity as a Measure of Hydration Capacity and the Relation of the Hydrogen Ion Concentration to Imbibition in the Different Acids, *J. Phys. Chem.*, 27: 481–492 (1923).

ners yield curves of essentially the same general shape. It is obvious that the normality of the acid which was added was not the true variable affecting the system, but that the hydrogen-ion concentration was in reality the variable concerned.

Another illustration of the importance of *p*H in biological problems is shown in Fig. 36, taken from the paper of Fife and Frampton,[13] who found that there is a hydrogen-ion concentration gradient in the leaf cells of the sugar beet and that the cells of the phloem are decidedly more basic than the parenchyma tissue. Leaf hoppers which transmit the virus of the curly-top disease normally feed on the sap of the phloem, and their saliva has approximately the same *p*H as normal phloem sap. When the *p*H of the phloem sap is altered by treatment

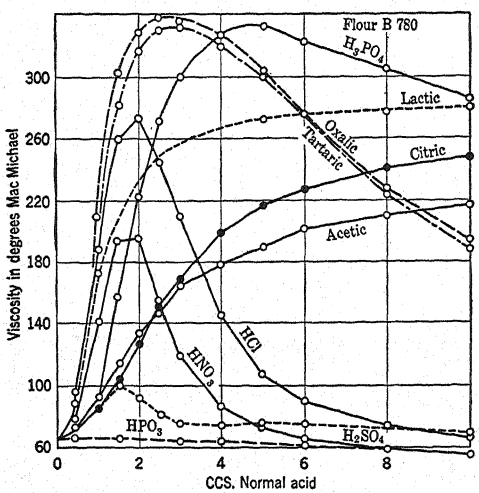


FIG. 34.—Showing the viscosity of flour-in-water systems plotted against the normality of solutions of the various acids. (Data of Gortner and Sharp.)

of the leaves with carbon dioxide, the leaf hoppers lose their sense of direction, their mouth parts do not penetrate into the phloem, and little or no infection with curly-top virus occurs.

It is beyond the scope of this book to more than mention the methods by which hydrogen-ion concentration can be measured. As noted at the beginning of this chapter, the excellent manuals of Clark and Michaelis are adequate in all respects. The two methods generally employed are colorimetric measurements and electrometric measurements.

[13] Fife, J. M., and Frampton, V. L., The *p*H Gradient Extending from the Phloem into the Parenchyma of the Sugar Beet and Its Relation to the Feeding Behavior of *Eutettix tenellus*, *J. Agr. Research*, 53: 581–593 (1936).

COLORIMETRIC MEASUREMENTS.—In the measurement of hydrogen-ion concentration by colorimetric methods one is concerned with the change in color of an indicator as a measurement of the change in hydrogen-ion concentration. Indicators are in general compounds which form salts with either acids or bases and yield on dissociation at least one colored ion. Accordingly the degree of dissociation of the indicator is altered by changes in the hydrogen- or hydroxyl-ion concentration. The concentration of the colored ion is accordingly altered, thus causing a change in the depth of color of the indicator as measured in a colorimeter. Various indicators have been suggested for the

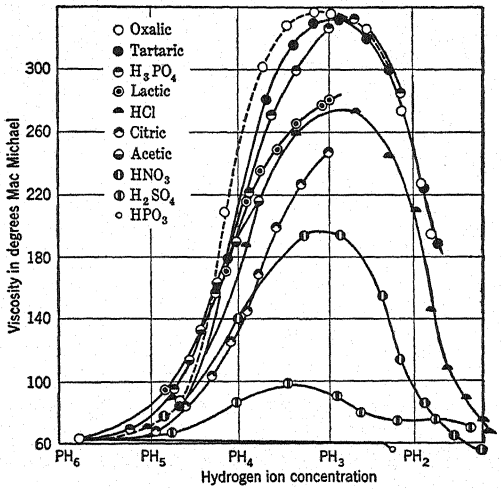


FIG. 35.—Showing the same data as in Fig. 34, plotted in terms of actual hydrogen-ion concentration.

various *p*H ranges. Table XIV shows the indicators which are most suitable, together with their color changes from acid to base and the *p*H range in which they can be used.

The phenolsulfonphthalein group of indicators are triphenyl methane derivatives containing a sulfophenyl and two phenol radicals, the latter being attached in their para positions to the methane carbon,

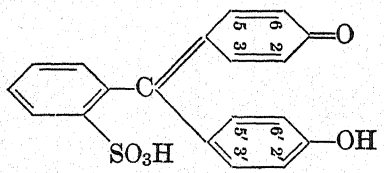


TABLE XIV

INDICATORS RECOMMENDED FOR THE COLORIMETRIC DETERMINATION OF HYDROGEN-ION CONCENTRATION TOGETHER WITH THEIR USABLE *p*H RANGE AND CERTAIN OTHER PHYSICO-CHEMICAL CONSTANTS *

Chemical Name	Common Name	Molecular Weight	*A*	*pK*	Range	Color Change		*B*	*C*	Absorption Max.	
						Acid	Alkaline			Acid	Alkaline
					*p*H			*p*H	*p*H	mμ	mμ
3.3′ dimethyl phenolsulfonphthalein	Meta cresol purple	382	26.2	1.51	1.2–2.8	Red	Yellow	conc. HCl	6	533	
2.2′ isopropyl 5.5′ dimethyl phenolsulfonphthalein	Thymol blue	466	21.5	1.5	1.2–2.8	Red	Yellow	conc. HCl	6	544	
2.2′ 6.6′ tetra brom phenolsulfonphthalein	Brom phenol blue	669	14.9	3.98	3.0–4.6	Yellow	Blue	0	7	.....	592
2.2′ 6.6′ tetra brom 3.3′ dimethyl phenolsulfonphthalein	Brom cresol green	698	14.3	4.67	3.8–5.4	Yellow	Blue	1	8	.....	617
2.2′ dichlor phenolsulfonphthalein	Chlor phenol red	423	23.6	5.98	4.8–6.4	Yellow	Red	2	9	.....	573
Ortho carboxy benzene azo dimethyl aniline	Methyl red	279	35.6	.....	4.4–6.0	Red	Yellow				
2.2′ dibrom phenolsulfonphthalein	Brom phenol red	512	19.5	6.16	5.2–6.8	Yellow	Red	3	10	.....	574
6.6′ dibrom 2.2′ dimethyl phenolsulfonphthalein	Brom cresol purple	540	18.5	6.3	5.2–6.8	Yellow	Purple	3	10	.....	591
6.6′ dibrom 2.2′ isopropyl 5.5′ methyl phenolsulfonphthalein	Brom thymol blue	624	16.0	7.0	6.0–7.6	Yellow	Blue	4	10	.....	617
Phenolsulfonphthalein	Phenol red	354	28.2	7.9	6.8–8.4	Yellow	Red	5	11	.....	558
2.2′ dimethyl phenolsulfonphthalein	Cresol red	382	26.2	8.3	7.2–8.8	Yellow	Red	5	11	.....	572
3.3′ dimethyl phenolsulfonphthalein	Meta cresol purple	382	26.2	8.32	7.4–9.0	Yellow	Purple	5	11	.....	580
2.2′ isopropyl 5.5′ dimethyl phenolsulfonphthalein	Thymol blue	466	21.5	8.9	8.0–9.6	Yellow	Blue	6	12	.....	596
Ortho cresol phthalein	Cresol phthalein	.....	.....	(9.4)	8.2–9.8	Colorless	Red	6	12		

A = cubic centimeters of 0.01 *N* NaOH required per 0.1 gram indicator to form mono sodium salt. Dilute to 250 cc. for 0.04 per cent reagent.
B = approximate *p*H value of solution required for full "acid color" appertaining to *p*H range indicated.
C = approximate *p*H value of solution required for full "alkaline color" appertaining to *p*H range indicated.
* From W. M. Clark, "The Determination of Hydrogen Ions, Third Edition," Williams and Wilkins Company, Baltimore. 1928. By permission.

Figure 37 [14] shows the dissociation curve of the indicators noted in Table XIV, the heavily shaded portion of the curve representing the range over which the indicator is useful. The indicator is in general most useful when it is approximately 50 per cent dissociated.

ELECTROMETRIC MEASUREMENTS. (*a*) *The Hydrogen Electrode.*—Electrometric determinations are based on the assumption that ions in solution obey the gas laws. If we allow a metal electrode to dip into a solution containing ions of that metal, the partial pressure of the ions in solution will be proportional to their ionic concentration. If the

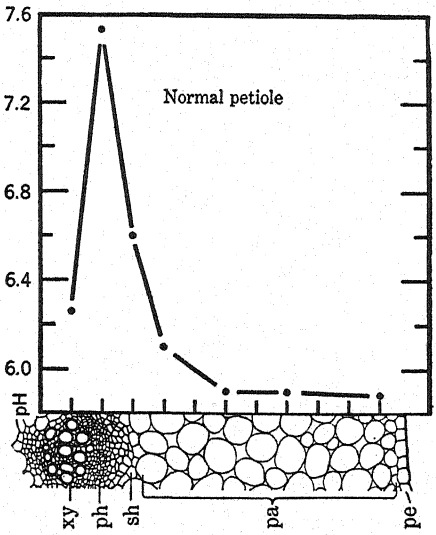


FIG. 36.—Showing curve of *p*H gradient in the normal petiole cells of the sugar beet. (Data of Fife and Frampton.)

electrode is of such size that one gram molecule of ions carrying nF faraday of electricity passes from the electrode to the solution, the partial pressure of the solution is raised by the amount dP, and a difference in potential is observed between the electrode and the solution which is equal to dE. The electrical energy which is expended will then be $nFdE$, and the work done upon the system will be VdP. Assuming that the process is reversible, the system comes to equilibrium where the amount of energy expended is equal to the amount of work done. Accordingly,

$$nFdE - VdP = 0 \qquad (33)$$

[14] From Clark, W. Mansfield, The Determination of Hydrogen Ions, Third Edition, Williams and Wilkins Company, Baltimore (1928). Reprinted by permission.

and from the gas laws $VP = RT$, or $V = \frac{RT}{P}$, we can write,

$$dE = \frac{RT}{nF} \cdot \frac{dP}{P} \tag{34}$$

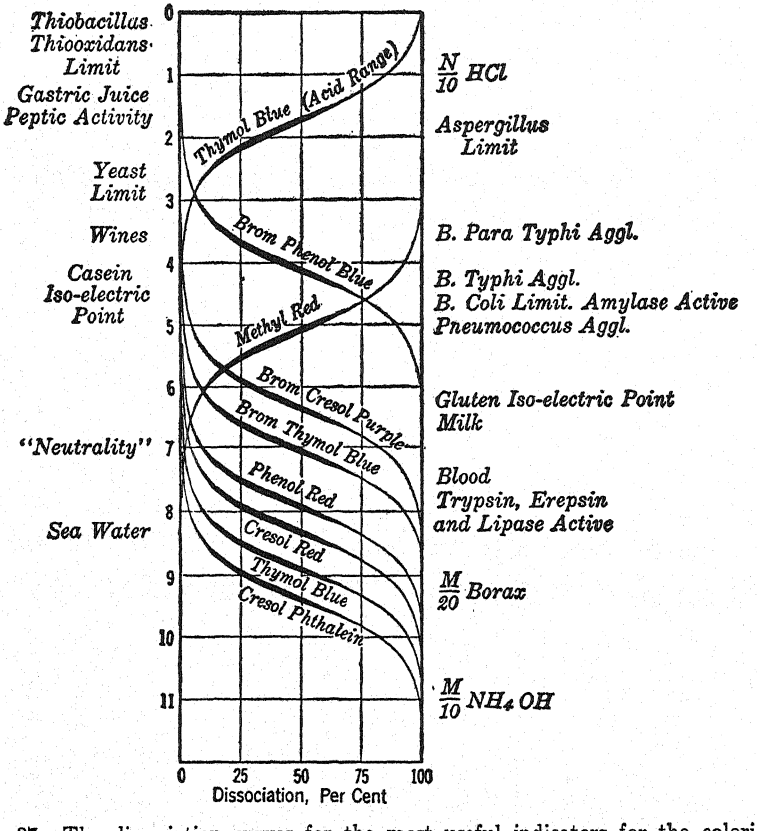


FIG. 37.—The dissociation curves for the most useful indicators for the colorimetric estimation of hydrogen-ion concentration. The shaded areas indicate the useful range. (From Clark's "Determination of Hydrogen Ions." By permission.)

This by integration becomes

$$E = \frac{RT}{nF} \log_e P + K \tag{35}$$

where $K =$ an integration constant.

The integration constant is the difference in potential between

the electrode and the solution when the pressure differs by some arbitrary unit. The Nernst equation, expressing the above, can be written

$$E = \frac{RT}{nF} \log_e \frac{P}{p} \qquad (36)$$

If we have two vessels arranged as in Fig. 38, the liquids in the two vessels differing in concentration, and these are connected in the manner shown in the figure, there will be set up a difference in potential between the two electrodes which can be detected by use of a suitable measuring device. Electrode 1 will have a potential $E = \frac{RT}{nF} \log_e P + K$, and electrode 2 a potential of $E' = \frac{RT}{nF} \log_e P' + K$, the potentials accordingly varying in direct relation to the osmotic pressure or to the concentration of ions in the solution. If P' is less than P, the electrode E' will be negative to the electrode E. The electromotive force which is set up can be expressed as

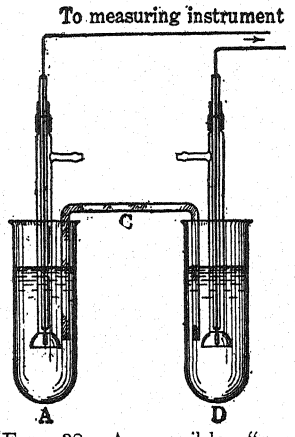


FIG. 38.—A possible "gas chain" arrangement in which A is the standard solution, D the test solution, and C a connection tube filled with an agar gel in a neutral, conducting solution (*e.g.*, KCl solution). The hydrogen electrode potential in A remains fixed, that in D varies with the acidity of the solution in D. (After Klopsteg.)

$$\text{e.m.f.} = E - E' \qquad (37)$$

or

$$\text{e.m.f.} = \left(\frac{RT}{nF} \log_e P + K\right) - \left(\frac{RT}{nF} \log_e P' + K\right) \qquad (38)$$

or

$$\text{e.m.f.} = \frac{RT}{nF} \log_e \frac{P}{P'} \qquad (39)$$

and since the osmotic pressures are proportional to the ion concentrations, it may be further written as

$$\text{e.m.f.} = \frac{RT}{nF} \log_e \frac{C}{C'} \qquad (40)$$

where C and C' represent the concentrations of the ions in the two solutions in question.

R = the gas constant;
T = the absolute temperature;
n = the valence of the ion;
F = the faraday or 96,500 coulombs.

The hydrogen electrode is nothing more than a metallic electrode which has been coated with a thin layer of platinum black deposited on an inactive metal and exposed to an atmosphere of hydrogen sufficiently long for the platinum black to become completely saturated with hydrogen.[15] When such an electrode is placed in a solution containing hydrogen ions, it reaches an equilibrium with the solution which varies with the concentration of the hydrogen ions in the solution, and behaves as if it were an electrode composed of metallic hydrogen. Figure 39 shows the electrode potential of the hydrogen

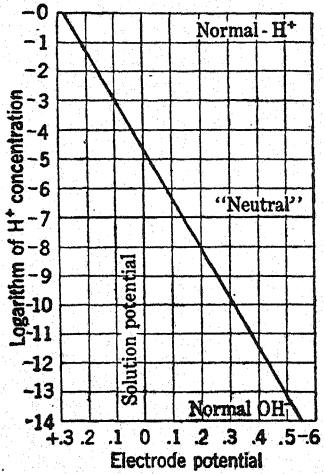


FIG. 39.

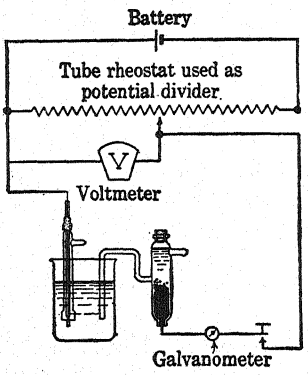


FIG. 40.

FIG. 39.—The electrode potential of the hydrogen electrode with reference to the solution in which it is immersed, plotted against the negative logarithm of the hydrogen-ion concentration. (After Klopsteg.)

FIG. 40.—Showing the connections for measuring gas-chain voltage. The e.m.f. from the gas chain is balanced by adjustment of the potential divider until the galvanometer shows zero. The voltmeter, permanently connected as shown, then reads the potential difference which was required to balance the unknown e.m.f. (After Klopsteg.)

electrode with reference to the solution in which it is immersed, when plotted against the negative logarithm of the hydrogen-ion concentration of the solution.

It is usually more convenient to alter the arrangement from that shown in Fig. 38, where two hydrogen electrodes are employed, by replacing one of the hydrogen electrodes with a calomel electrode (the so-called calomel half cell) which can be made up to have a fixed po-

[15] *Cf.* Kolthoff, I. M., and Kameda, T., The Measurement of the Hydrogen Ion Concentration in Unbuffered Solutions. I. The Adsorbent Properties of Platinized Platinum, *J. Am. Chem. Soc.*, 51: 2888–2900 (1929).

tential. Such an arrangement is shown in Fig. 40. Equation (40) may be rewritten in terms of one hydrogen electrode immersed in a solution containing a normal concentration of hydrogen ions as a reference electrode, and an electrode immersed in a solution containing an unknown concentration of hydrogen ions (C_a), the concentration of which it is desirable to determine. The equation then becomes

$$E = 0.00019837T \log \frac{1}{C_a} \qquad (41)\ ^{16}$$

providing that the concentration of hydrogen ions in the solution of C_a is less than a normal solution. It is very difficult to prepare a solution *exactly* normal with respect to hydrogen ions, and though such a solution has been used as the ultimate standard, the practical reference electrode is the so-called calomel electrode. Equation (41), when the calomel electrode is used, therefore becomes

$$\frac{\text{e.m.f. (observed)} - E\text{ (calomel electrode)}}{0.00019837T} = \log \frac{1}{[H^+]} = p\text{H} \qquad (42)$$

which gives the hydrogen-ion concentration directly in pH.

Schmidt and Hoagland [17] have placed in convenient form the pH, C_H and C_{OH} values corresponding to the various millivolt readings obtained when the normal calomel or the 0.1 N calomel electrode is used as the reference standard. Their values have been calculated by substituting the appropriate values in the above equation.

Lewis, Brighton, and Sebastion [18] give the value of $E = -0.2828$, as the potential of the normal calomel electrode. Guggenheim and Schindler [19] report a value of $E = -0.3337$ volt at 25° C. for the 0.1 N calomel electrode.

Figures 38, 39, and 40 are taken from a paper by Klopsteg [20] where an excellent discussion of the principles involved in the electrometric measurement of hydrogen-ion concentration is given. McClendon [21] has also presented an excellent discussion of the principles involved in such measurements, with particular reference to biological applications.

[16] In this equation the logarithm is referred to the base, 10.

[17] Schmidt, C. L. A., and Hoagland, D. R., Table of P_H, H^+, and OH^- Values Corresponding to Electromotive Forces Determined in Hydrogen Electrode Measurements, with a Bibliography, *Univ. of California Publications in Physiol.*, **5** (No. 4): 23–69 (1919).

[18] Lewis, G. N., Brighton, T. B., and Sebastion, R. L., A Study of Hydrogen and Calomel Electrodes, *J. Am. Chem. Soc.*, 39: 2245–2261 (1917).

[19] Guggenheim, E. A., and Schindler, T. D., Studies of Cells with Liquid-Liquid Junctions V. Standards for Hydrogen Ion Measurements and the Second Dissociation Constant of Phosphoric Acid, *J. Phys. Chem.*, 38: 533–541 (1934).

[20] Klopsteg, P. E., Some Practical Aspects of Hydrogen Electrode Measurements, *J. Ind. Eng. Chem.*, 14: 399-406 (1922).

[21] McClendon, J. F., Hydrogen and Hydroxyl Ion Concentration in Physiology and Medicine, *Med. Rev. Rev.*, 22: 333-365 (1916).

Electrodes other than the normal calomel electrode may be employed advantageously in certain instances. Gerke [22] gives a summary of electrode potentials, and Sharp and MacDougall [23] described the preparation of a series of electrodes which can be used in titrating solutions to definite *p*H values. Figure 41, taken from their paper, indicates the set-up which is involved. *B* is a dipping hydrogen electrode; siphon *C* and vessel *D* contain a saturated solution of potassium chloride. The liquid to be titrated is placed in beaker *A*, and connection is made with the reference electrode *G*, as shown in the diagram. Acid or alkali is added from a buret to the liquid in beaker *A* until no deflection is noted on the galvanometer or electrometer, *i.e.*, until no current flows between the reference electrode and the hydrogen electrode. When such an equilibrium is reached, the liquid in beaker *A* is at a definite *p*H value depending upon the reference electrode which is used at *G*. Table XV gives the composition of their reference electrodes. The last column in the table indicates the *p*H of the solution in beaker *A* at the time that no current flows through the galvanometer.

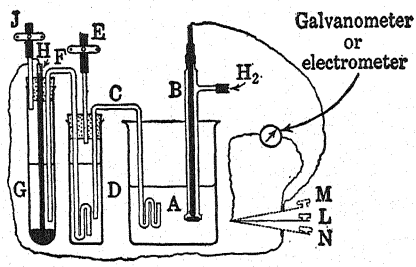
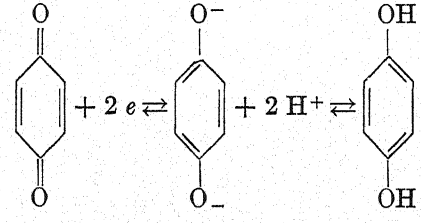


FIG. 41.—Showing the connections necessary to determine the *p*H of an unknown system, using the special electrode half-cells of Sharp and MacDougall.

(*b*) *The Quinhydrone Electrode.*—Quinhydrone is an equimolecular mixture of quinone and hydroquinone. Quinone may be regarded as the oxidation product of hydroquinone. The reactions which probably take place are the addition of two electrons to quinone to form the anion of hydroquinone, and this anion reacts with two hydrogen ions to form hydroquinone:



Quinone — Anion of Hydroquinone — Hydroquinone

[22] Gerke, R. H., A. Summary of Electrode Potentials, *Chem. Rev.*, 1: 377–395 (1925).

[23] Sharp, P. F., with MacDougall, F. H., A Simple Method of Electrometric Titration in Acidimetry and Alkalimetry, *J. Am. Chem. Soc.*, 44: 1193–1196 (1922).

TABLE XV

COMPOSITION OF SPECIAL ELECTRODES TO BE USED AS HALF CELLS IN TITRATING SOLUTIONS TO DEFINITE *p*H VALUES

Electrode Solution. Dilute Quantities Given to Total Volume of 100 cc.				Potential against Normal Calomel Electrode	Calculated Potential of Hydrogen Electrode in Solution of Indicated *p*H	*p*H
	cc.	*N*	Salt			
12–12.5% Lead Amalgam and Lead Iodide	0.52	2.0	KI	0.5195	0.5194	4.0
	2.90	2.0	KI	0.5609	0.5608	4.7
	6.14	2.0	KI	0.5786	0.5786	5.0
	67.50	2.0	KI	0.6378	0.6377	6.0
12–12.5% Cadmium Amalgam	100.00	0.5	$CdSO_4$	0.6967	0.6968	7.0
	0.20	2.0	KI			
	10.00	0.5	$CdSO_4$	0.7560	0.7560	8.0
	10.14	2.0	KI			
	100.00	0.5	$CdSO_4$	0.8150	0.8151	9.0
	26.40	2.0	KI			
	2.00	0.5	$CdSO_4$	0.8742	0.8743	10.0
	48.70	2.0	KI			

The development of this electrode is largely due to the work of Biilmann [24, 25] and his co-workers.

Since the reaction is in reality

$$\frac{[\text{quinone}][H^+]^2[e]^2}{[\text{hydroquinone}]^-} = K, \tag{43}$$

this can be expressed as

$$E_h = E_0 - \frac{RT}{2F} \log_e \frac{[\text{hydroquinone}]}{[\text{quinone}]} + \frac{RT}{F} \log_e [H^+] \tag{44}$$

where E_h = the observed potential referred to the normal hydrogen electrode;

E_0 = the characteristic constant of the system.

Since the solution is kept saturated with quinhydrone, we do not need to consider the amounts of quinone or hydroquinone which are present in the system. Equation (44) accordingly reduces to

$$E_h = E_q + \frac{RT}{F} \log_e [H^+] \tag{45}$$

[24] Biilmann, E., L'électrode à quinhydrone et ses applications, *Bull. soc. chim.*, (4) 41: 213–286 (1927).

[25] Biilmann, E., and Jensen, A. L., Sur le potentiel de l'électrode à quinhydrone par rapport à l'électrode à hydrogène, *Bull. soc. chim.*, (4) 41: 151–166 (1927).

where E_q = the potential of the quinone-hydroquinone system. At 25° C. this equation reduces to

$$E_h = E_q - 0.05912\ p\mathrm{H} \tag{46}$$

The quinhydrone electrode is in reality an oxidation-reduction system, and the potential which is developed is in reality an oxidation-reduction potential (*vide infra*) as modified by changes in hydrogen-ion concentration. It should be emphasized therefore that *the quinhydrone electrode can give accurate results for hydrogen-ion concentration only when no other oxidation-reduction potential is present in the system.* In other words, if oxidases are present and if organic compounds are present which can be acted upon by oxidases, such an enzyme substrate system will give rise to an independent oxidation-reduction potential of an unknown magnitude which will be impressed upon the oxidation-reduction potential of the quinhydrone system and accordingly may give potentials which are not related to the hydrogen-ion concentration of the system under investigation. This possibility must be constantly kept in mind in applying the quinhydrone technic to the study of hydrogen-ion concentration in biological systems. For a further discussion of the quinhydrone system the reader is referred to Clark's monograph.

(*c*) *The Glass Electrode.*—The glass electrode is rapidly replacing the hydrogen electrode for the determination of hydrogen-ion concentration. Haber and Klemensiewicz [26] introduced the glass electrode by inserting a thin glass membrane at the boundary A in the following concentration cell,

$$\mathrm{Hg} \mid \mathrm{HgCl},\ \mathrm{KCl} \mid \text{Solution I} \underset{A}{\mid} \text{Solution II} \mid \mathrm{KCl},\ \mathrm{HgCl} \mid \mathrm{Hg}$$

where Solution I and Solution II differ from each other in hydrogen-ion concentration. If the membrane A in the above concentration cell was composed of a thin sheet of silver foil and Solutions I and II were solutions of a silver salt differing only in silver-ion concentration, we would find that such an arrangement would deliver a potential proportional to the difference in the concentration of the silver ions.

$$E = \frac{RT}{F} \log_e \frac{[\mathrm{Ag}^+]_1}{[\mathrm{Ag}^+]_2} \tag{47}$$

Haber and Klemensiewicz considered that glass might be regarded as a phase containing water, hydrogen, and hydroxyl ions at constant concentration. Accordingly equation (47) would be stated as

$$E = \frac{RT}{F} \log_e \frac{[\mathrm{H}^+]_1}{[\mathrm{H}^+]_2} \tag{48}$$

[26] Haber, F., and Klemensiewicz, Z., Über elektrische Phasengrenzkräfte, *Z. physik. Chem.*, 67: 385–431 (1909).

Radio amplification has extended the applicability of the glass electrode to the use of glass membranes which are relatively thick and relatively rugged. The Beckman, the Coleman, and the Hellige *p*H electrometers are common examples of instruments in which the glass electrode is incorporated. Micro forms of glass electrodes have been constructed which enable the accurate determination of the hydrogen-ion concentration in as little as 0.1 cc. of biological fluids, and the glass electrode has the further advantage in that there is no contamination of the biological fluid with chemicals, so that, after the determination of *p*H, the fluid can be used for other analytical procedures.

BUFFERS.—Van Slyke [27] has defined buffers as *"substances which by their presence in solution increase the amount of acid or alkali that must be added to cause unit change in pH."* The most efficient buffers are mixtures of weak acids or weak bases with their corresponding salts.

As noted above, a weak acid or a weak base is characterized by the fact that a large proportion of the material in solution is present in the form of undissociated molecules, and accordingly the dissociation constant is low. In acetic acid only a small fraction of the potential acidity is present at any one time, the dissociation constant having a value of approximately 1.8×10^{-5}. The equilibrium equation can therefore be written

$$\frac{[H^+] \times [C_2H_3O_2^-]}{[HC_2H_3O_2]} = 1.8 \times 10^{-5}$$

The value 1.8×10^{-5} is constant regardless of the concentration of acetic acid in the solution. If more acetic acid is added to the solution, the concentration of undissociated molecules is increased and a small part of the added acetic acid dissociates to maintain the above equilibrium. If another substance with a common ion, for example, sodium acetate, is added, the concentration of the added acetate ions must be taken into consideration in the above equation, and since this would cause an increase in the anion portion of the numerator, there would be a corresponding decrease in the hydrogen-ion concentration.

Buffers may also be defined as *those substances which prevent sudden or great changes in hydrogen-ion concentration when strong acids or bases are added to a system.* If one adds hydrochloric acid to a solution of trisodium phosphate, the following reactions take place,

$$Na_3PO_4 + HCl = NaCl + Na_2HPO_4$$
$$Na_2HPO_4 + HCl = NaCl + NaH_2PO_4$$
$$NaH_2PO_4 + HCl = NaCl + H_3PO_4$$

[27] Van Slyke, D. D., On the Measurement of Buffer Values and on the Relationship of Buffer Value to the Dissociation Constant of the Buffer and the Concentration and Reaction of the Buffer Solution, *J. Biol. Chem.*, 52: 525–570 (1922).

Assuming that the original solution of trisodium phosphate is a 0.3 N solution and that one equivalent of hydrochloric acid is added to it, the solution of disodium phosphate which results from the adding of the strongly acid hydrochloric acid (pH = 1) would not only be not acid but would actually be alkaline and would have a pH of approximately 8.5 to 9.

If, to this slightly alkaline solution, we again add an equivalent of hydrochloric acid, the resulting monosodium phosphate solution would have a pH of approximately 4.5, and if to this solution of monosodium phosphate a third equivalent of hydrochloric acid is added, the resulting solution of phosphoric acid would have a pH of approximately 2.5. Three equivalents of hydrochloric acid, having a pH of 1.0, have thus been needed to shift the hydrogen-ion concentration of trisodium phosphate from approximately pH = 10.5 to approximately pH = 2.5.

In a similar way the reaction between sodium acetate and hydrochloric acid may be written

$$NaC_2H_3O_2 + HCl = NaCl + H \cdot C_2H_3O_2$$

and in this case the hydrochloric acid solution having a pH of 1.0 has been "buffered" by the sodium acetate, so that the resulting mixture has a pH of approximately 2.5. Sodium carbonate, likewise, acts as a buffer according to the following reaction:

$$Na_2CO_3 + HCl = NaCl + NaHCO_3$$

and on the addition of a second equivalent of hydrochloric acid,

$$NaHCO_3 + HCl = NaCl + H_2CO_3$$

Carbonic acid is such a weak acid that it decomposes according to the following equation:

$$H_2CO_3 = CO_2 + H_2O$$

and the carbon dioxide may be given off from the solution in the form of gas bubbles. Thus, we have here conditions where two equivalents of a strong acid can be added to a slightly alkaline solution, with a resulting solution which is essentially neutral. *This is one of the reactions by which the animal body is protected against sudden changes in hydrogen-ion concentration.* The blood contains a considerable amount of bicarbonate. If diluted hydrochloric acid is injected intravenously, the acid is buffered by the bicarbonate of the blood stream; the carbon dioxide which is formed is eliminated by the lungs; the hydrochloric acid is converted into sodium chloride, a normal constituent of the blood; a part of the remaining bicarbonate dissociates so as to keep a constant K_b; and there is no appreciable change in the

hydrogen-ion concentration of the blood stream. Very considerable amounts of strong acids can accordingly be neutralized by the body through the action of the normal buffers of the blood and tissues. If, however, we were to continue the injection of dilute hydrochloric acid until the buffers in the blood stream had been exhausted, there would suddenly occur a marked rise in the hydrogen-ion concentration of the blood, a violent physiological reaction, and if sufficient hydrochloric acid were injected, death would ensue. *All reactions of living protoplasm take place in buffered media.* Carbonates, bicarbonates, and phosphates are the principal buffers met with in biological processes, although proteins may under certain conditions act as relatively inefficient buffers.

The same sort of reactions which take place in the buffering of a strong acid are involved when strong bases are employed. Thus, for example, ammonium acetate will act as a buffer for sodium hydroxide according to the following reaction:

$$NH_4C_2H_3O_2 + NaOH = NaC_2H_3O_2 + NH_4OH$$

where the strong base, sodium hydroxide, has been buffered and replaced by the weak ammonium hydroxide, or, using a phosphate,

$$NaH_2PO_4 + NaOH = Na_2HPO_4 + H_2O$$

Most biological reactions take place in an essentially neutral or slightly acid medium, and as a rule biological organisms have a greater capacity for the buffering of acid solutions than for the buffering of bases.

An apparent exception to the general rule that the tissues of living organisms are characterized by hydrogen-ion concentrations in the region of neutrality is afforded by the observations of Kobayashi,[28] who studied the blood plasma and corpuscles of the ascidian, *Chelyosoma siboja.* He found that the body fluid had a *p*H of 1.54, the plasma a *p*H of 1.80, and the corpuscle fluid a *p*H of 0.38. These high acidities were due to the presence of free sulfuric acid which ranged from an SO_4^- concentration in the plasma from 3.95 to 6.53 grams per liter, in the body fluid from 8.33 to 23.50 grams per liter, and in the corpuscle fluid from 42.4 to 55.29 grams per liter, making concentrations of approximately 0.88 *N* in the corpuscle fluid, 0.36 *N* in the body fluid, and 0.027 *N* in the plasma. Further biochemical and physiological studies on such an unusual condition in living organisms are highly to be desired.

[28] Kobayashi, S., Acid of the Body Fluid from an Ascidian, *Chelyosoma siboja Oka., Science Repts. Tohoku Imp. Univ.* (4th Ser.), 8: 277–285 (1933). *cf.* also Henze, M., *Z. physiol. Chem.*, 72: 494–501 (1911); 79: 215–228 (1912); 86: 340–344 (1913); Hecht, S., *Am. J. Physiol.*, 45: 157–187 (1917).

Van Slyke [29] has proposed a unit for the measurement of buffering values. The unit adopted is the differential ratio, $\frac{dB}{dpH}$, which expresses the relationship between the increment in gram equivalents per liter of a strong base (B) added to a buffer solution, and the resultant increment in pH. Correspondingly for the acid range the increment of strong acid is equivalent to a negative increment of the base ($-dB$). In these terms a solution has a buffer value of 1.0 when a liter will take up a gram equivalent of strong acid or alkali per unit change in pH.

As Van Slyke points out, if a base is added to a solution, the pH is increased so that both dB and dpH are positive. If an acid is added,

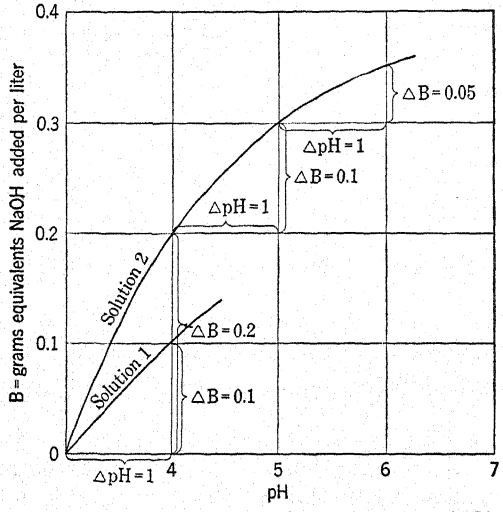


FIG. 42.—Showing the relationship between buffer capacity (ΔB) and change in hydrogen-ion concentration. (Data of Van Slyke.)

dB and dpH both are negative. However, the ratio $\frac{dB}{dpH}$ always has a positive numerical value. If one solution has twice the buffer value of a second solution, it will require twice as much acid or base to change the pH of the former through a unit range. The value of $\frac{dB}{dpH}$ is therefore twice as great for the first solution as for the second. Van Slyke suggests that the symbol β be used to indicate the ratio $\frac{dB}{dpH}$. Figure 42,

[29] Van Slyke, D. D., On the Measurement of Buffer Values and on the Relationship of Buffer Value to the Dissociation Constant of the Buffer and the Concentration and Reaction of the Buffer Solution, *J. Biol. Chem.*, 52: 525-570 (1922).

taken from the paper by Van Slyke, represents the buffer value of the two solutions referred to in this paragraph.

It would take us too far afield to consider adequately the various factors which must be taken into consideration in a study of buffer action. The manuals by Clark and Michaelis are adequate for a general understanding of the principles which are involved, and the paper by Van Slyke which is noted above gives an extended discussion of the theories which are involved. The average biochemist or biologist is usually concerned not so much with the theory underlying the general principle as he is with the practical application of the principle to laboratory problems. Perhaps the greatest application of the principles of buffer action, aside from the interpretation of the resistance of living organisms to changes in acidity or alkalinity, lies in the ability to prepare from known mixtures of chemicals, solutions which have a definite and stable *p*H value. Table XVI lists the composition of buffer mixtures having a given *p*H value at 20° C.

TABLE XVI

COMPOSITION OF MIXTURES GIVING *p*H VALUES AT 20° C. AT INTERVALS OF 0.2 *

KCL–HCl Mixtures †

*p*H			
1.2	50 cc. *M*/5 KCl	65.5 cc. *M*/5 HCl	Dilute to 200 cc.
1.4	50 cc. *M*/5 KCl	41.5 cc. *M*/5 HCl	Dilute to 200 cc.
1.6	50 cc. *M*/5 KCl	26.3 cc. *M*/5 HCl	Dilute to 200 cc.
1.8	50 cc. *M*/5 KCl	16.6 cc. *M*/5 HCl	Dilute to 200 cc.
2.0	50 cc. *M*/5 KCl	10.6 cc. *M*/5 HCl	Dilute to 200 cc.
2.2	50 cc. *M*/5 KCl	6.7 cc. *M*/5 HCl	Dilute to 200 cc.

Phthalate–HCl Mixtures

2.2	50 cc. *M*/5 KH Phthalate	46.70 cc. *M*/5 HCl	Dilute to 200 cc.
2.4	50 cc. *M*/5 KH Phthalate	39.60 cc. *M*/5 HCl	Dilute to 200 cc.
2.6	50 cc. *M*/5 KH Phthalate	32.95 cc. *M*/5 HCl	Dilute to 200 cc.
2.8	50 cc. *M*/5 KH Phthalate	26.42 cc. *M*/5 HCl	Dilute to 200 cc.
3.0	50 cc. *M*/5 KH Phthalate	20.32 cc. *M*/5 HCl	Dilute to 200 cc.
3.2	50 cc. *M*/5 KH Phthalate	14.70 cc. *M*/5 HCl	Dilute to 200 cc.
3.4	50 cc. *M*/5 KH Phthalate	9.90 cc. *M*/5 HCl	Dilute to 200 cc.
3.6	50 cc. *M*/5 KH Phthalate	5.97 cc. *M*/5 HCl	Dilute to 200 cc.
3.8	50 cc. *M*/5 KH Phthalate	2.63 cc. *M*/5 HCl	Dilute to 200 cc.

* From W. M. Clark, "The Determination of Hydrogen Ions," Williams and Wilkins Company, Baltimore. 1928. By permission.

† The *p*H values of these mixtures are given by Clark and Lubs (1916) as *preliminary* measurements.

TABLE XVI—*Continued*

Phthalate–NaOH Mixtures

*p*H			
4.0	50 cc. *M*/5 KH Phthalate	0.40 cc. *M*/5 NaOH	Dilute to 200 cc.
4.2	50 cc. *M*/5 KH Phthalate	3.70 cc. *M*/5 NaOH	Dilute to 200 cc.
4.4	50 cc. *M*/5 KH Phthalate	7.50 cc. *M*/5 NaOH	Dilute to 200 cc.
4.6	50 cc. *M*/5 KH Phthalate	12.15 cc. *M*/5 NaOH	Dilute to 200 cc.
4.8	50 cc. *M*/5 KH Phthalate	17.70 cc. *M*/5 NaOH	Dilute to 200 cc.
5.0	50 cc. *M*/5 KH Phthalate	23.85 cc. *M*/5 NaOH	Dilute to 200 cc.
5.2	50 cc. *M*/5 KH Phthalate	29.95 cc. *M*/5 NaOH	Dilute to 200 cc.
5.4	50 cc. *M*/5 KH Phthalate	35.45 cc. *M*/5 NaOH	Dilute to 200 cc.
5.6	50 cc. *M*/5 KH Phthalate	39.85 cc. *M*/5 NaOH	Dilute to 200 cc.
5.8	50 cc. *M*/5 KH Phthalate	43.00 cc. *M*/5 NaOH	Dilute to 200 cc.
6.0	50 cc. *M*/5 KH Phthalate	45.45 cc. *M*/5 NaOH	Dilute to 200 cc.
6.2	50 cc. *M*/5 KH Phthalate	47.00 cc. *M*/5 NaOH	Dilute to 200 cc.

KH_2PO_4–NaOH Mixtures

*p*H			
5.8	50 cc. *M*/5 KH_2PO_4	3.72 cc. *M*/5 NaOH	Dilute to 200 cc.
6.0	50 cc. *M*/5 KH_2PO_4	5.70 cc. *M*/5 NaOH	Dilute to 200 cc.
6.2	50 cc *M*/5 KH_2PO_4	8.60 cc. *M*/5 NaOH	Dilute to 200 cc.
6.4	50 cc. *M*/5 KH_2PO_4	12.60 cc. *M*/5 NaOH	Dilute to 200 cc.
6.6	50 cc. *M*/5 KH_2PO_4	17.80 cc. *M*/5 NaOH	Dilute to 200 cc.
6.8	50 cc. *M*/5 KH_2PO_4	23.65 cc. *M*/5 NaOH	Dilute to 200 cc.
7.0	50 cc. *M*/5 KH_2PO_4	29.63 cc. *M*/5 NaOH	Dilute to 200 cc.
7.2	50 cc. *M*/5 KH_2PO_4	35.00 cc. *M*/5 NaOH	Dilute to 200 cc.
7.4	50 cc. *M*/5 KH_2PO_4	39.50 cc. *M*/5 NaOH	Dilute to 200 cc.
7.6	50 cc. *M*/5 KH_2PO_4	42.80 cc. *M*/5 NaOH	Dilute to 200 cc.
7.8	50 cc. *M*/5 KH_2PO_4	45.20 cc. *M*/5 NaOH	Dilute to 200 cc.
8.0	50 cc. *M*/5 KH_2PO_4	46.80 cc. *M*/5 NaOH	Dilute to 200 cc.

Boric Acid. KCl–NaOH Mixtures

*p*H			
7.8	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	2.61 cc. *M*/5 NaOH	Dilute to 200 cc.
8.0	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	3.97 cc. *M*/5 NaOH	Dilute to 200 cc.
8.2	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	5.90 cc. *M*/5 NaOH	Dilute to 200 cc.
8.4	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	8.50 cc. *M*/5 NaOH	Dilute to 200 cc.
8.6	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	12.00 cc. *M*/5 NaOH	Dilute to 200 cc.
8.8	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	16.30 cc. *M*/5 NaOH	Dilute to 200 cc.
9.0	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	21.30 cc. *M*/5 NaOH	Dilute to 200 cc.
9.2	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	26.70 cc. *M*/5 NaOH	Dilute to 200 cc.
9.4	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	32.00 cc. *M*/5 NaOH	Dilute to 200 cc.
9.6	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	36.85 cc. *M*/5 NaOH	Dilute to 200 cc.
9.8	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	40.80 cc. *M*/5 NaOH	Dilute to 200 cc.
10.0	50 cc. *M*/5 H_3BO_3, *M*/5 KCl	43.90 cc. *M*/5 NaOH	Dilute to 200 cc.

Sörenson [30] gives a series of buffer mixtures different from those suggested by Clark. The compositions of Sörenson's mixtures are shown in Table XVII. Temple [31] recommends sodium maleate as a very efficient buffer for the region 5.2–6.8.

TABLE XVII

COMPOSITION OF SÖRENSEN'S BUFFER MIXTURES

(*a*) Mixtures of Glycine * and Hydrochloric Acid †

Volume of Glycine Solution	Volume of HCl	*p*H of Mixture	Volume of Glycine Solution	Volume of HCl	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	....	6.106	6.00	4.00	2.279
9.90	0.10	4.411	5.00	5.00	1.932
9.75	0.25	3.991	4.00	6.00	1.645
9.50	0.50	3.679	3.00	7.00	1.419
9.00	1.00	3.341	2.00	8.00	1.251
8.00	2.00	2.922	1.00	9.00	1.146
7.00	3.00	2.607	....	10.00	1.038

* 7.505 grams of glycine and 5.85 grams NaCl in 1 liter of solution.
† An exactly 0.1 *N* solution of hydrochloric acid.

(*b*) Mixtures of Glycine * and Sodium Hydroxide †

Volume of Glycine Solution	Volume of NaOH	*p*H of Mixture	Volume of Glycine Solution	Volume of NaOH	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	....	6.106	5.10	4.90	11.067
9.90	0.10	7.809	5.00	5.00	11.305
9.75	0.25	8.237	4.90	5.10	11.565
9.50	0.50	8.575	4.50	5.50	12.095
9.00	1.00	8.929	4.00	6.00	12.399
8.00	2.00	9.364	3.00	7.00	12.674
7.00	3.00	9.714	2.00	8.00	12.856
6.00	4.00	10.140	1.00	9.00	12.972
5.50	4.50	10.482	....	10.00	13.066

* Same concentration as in Section (*a*) of this table..
† An exactly 0.1 *N* solution of carbonate-free NaOH

[30] Sörensen, S. P. L., Études enzymatiques. II. Sur la mesure et l'importance de la concentration des ions hydrogène dans les réactions enzymatiques, *Compt. rend. Trav. lab. Carlsberg*, 8: 41, 42, 43 (1909). Compare also Kolthoff, I. M., A New Set of Buffer Mixtures That Can Be Prepared Without the Use of Standardized Acid or Base, *J. Biol Chem.*, 63: 135–141 (1925).

[31] Temple, J. W., Sodium Maleate—A Buffer for the *p*H Region of 5.2–6.8, *J. Am. Chem. Soc.*, 51: 1754–1755 (1929).

TABLE XVII—*Continued*

(*c*) Mixtures of Sodium Monohydrogen Phosphate * and Potassium Dihydrogen Phosphate †

Volume of Na_2HPO_4 Solution	Volume of KH_2PO_4 Solution	*p*H of Mixture	Volume of Na_2HPO_4 Solution	Volume of KH_2PO_4 Solution	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	....	8.302	4.00	6.00	6.643
9.90	0.10	8.171	3.00	7.00	6.468
9.75	0.25	8.038	2.00	8.00	6.239
9.50	0.50	7.863	1.00	9.00	5.910
9.00	1.00	7.648	0.50	9.50	5.600
8.00	2.00	7.347	0.25	9.75	5.305
7.00	3.00	7.146	0.10	9.90	4.976
6.00	4.00	6.976	....	10.00	4.529
5.00	5.00	6.813			

* 11.876 grams $Na_2HPO_4 \cdot 2H_2O$ in 1 liter of solution.
† 9.078 grams KH_2PO_4 in 1 liter of solution.

(*d*) Mixtures of Sodium Borate* and 0.1 *N* Hydrochloric Acid

Volume of Borate Solution	Volume of HCl	*p*H of Mixture	Volume of Borate Solution	Volume of HCl	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	....	9.241	6.50	3.50	8.506
9.50	0.50	9.168	6.00	4.00	8.289
9.00	1.00	9.087	5.75	4.25	8.137
8.50	1.50	9.007	5.50	4.50	7.939
8.00	2.00	8.908	5.25	4.75	7.621
7.50	2.50	8.799	5.00	5.00	6.548
7.00	3.00	8.678	4.75	5.25	2.371

* 12.404 grams of boric acid dissolved in 100 cc. of exactly 1.0 *N* carbonate-free NaOH and diluting the mixture to 1 liter volume.

(*e*) Mixtures of Sodium Borate * and 0.1 *N* Sodium Hydroxide

Volume of Borate Solution	Volume of NaOH	*p*H of Mixture	Volume of Borate Solution	Volume of NaOH	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	....	9.241	6.00	4.00	9.974
9.00	1.00	9.360	5.00	5.00	11.076
8.00	2.00	9.503	4.00	6.00	12.376
7.00	3.00	9.676			

* Borate solution of same composition as in Section (*d*) of this table.

TABLE XVII—*Continued*

(*f*) Mixture of Sodium Citrate * and 0.1 *N* Hydrochloric Acid

Volume of Citrate Solution	Volume of HCl	*p*H of Mixture	Volume of Citrate Solution	Volume of HCl	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	....	4.958	4.75	5.25	3.529
9.50	0.50	4.887	4.50	5.50	3.364
9.00	1.00	4.830	4.00	6.00	2.972
8.00	2.00	4.652	3.33	6.67	2.274
7.00	3.00	4.447	3.00	7.00	1.925
6.00	4.00	4.158	2.00	8.00	1.418
5.50	4.50	3.948	1.00	9.00	1.173
5.00	5.00	3.692	....	10.00	1.038

* 21.008 grams of citric acid ($C_6H_8O_7 \cdot H_2O$) dissolved in 200 cc. of carbonate-free 1.0 *N* NaOH solution and the mixture diluted to 1 liter.

(*g*) Mixtures of Sodium Citrate * and 0.1 *N* Sodium Hydroxide

Volume of Citrate Solution	Volume of NaOH	*p*H of Mixture	Volume of Citrate Solution	Volume of NaOH	*p*H of Mixture
cc.	cc.		cc.	cc.	
10.00	....	4.958	5.50	4.50	6.331
9.50	0.50	5.023	5.25	4.75	6.678
9.00	1.00	5.109	5.00	5.00	9.052
8.00	2.00	5.314			10.092
7.00	3.00	5.568	4.50	5.50	12.073
6.00	4.00	5.969	4.00	6.00	12.364

* Same concentration as solution used in Section (*f*) of this table.

As noted above, proteins may act to a certain extent as buffers. Their efficiency as buffers depends to a very great extent on the chemical nature of the amino acids making up the protein molecule. Figure 43 [32] shows the change in hydrogen-ion concentration of two typical proteins when acid or alkali is added to the system. In the case of durumin, the prolamine of durum wheat, there is no evidence of buffer action toward sodium hydroxide, and not much evidence of buffer action toward hydrochloric acid. In the case of casein from milk there is a definite buffering of added sodium hydroxide in the range from

[32] Hoffman, W. F., and Gortner, R. A., Physico-chemical Studies on Proteins. I. The Prolamines—Their Chemical Composition in Relation to Acid and Alkali Binding, Colloid Symposium Monograph, Vol. II, pp. 209–368, Chemical Catalog Company, New York (1925).

approximately *p*H 6.0 to *p*H 7.0, with a slight but less-pronounced buffering effect against acid in the range of approximately *p*H 4.0 to *p*H 3.0. On the whole, however, proteins may be regarded as fairly inefficient buffers, and the generalization noted above that carbonates,

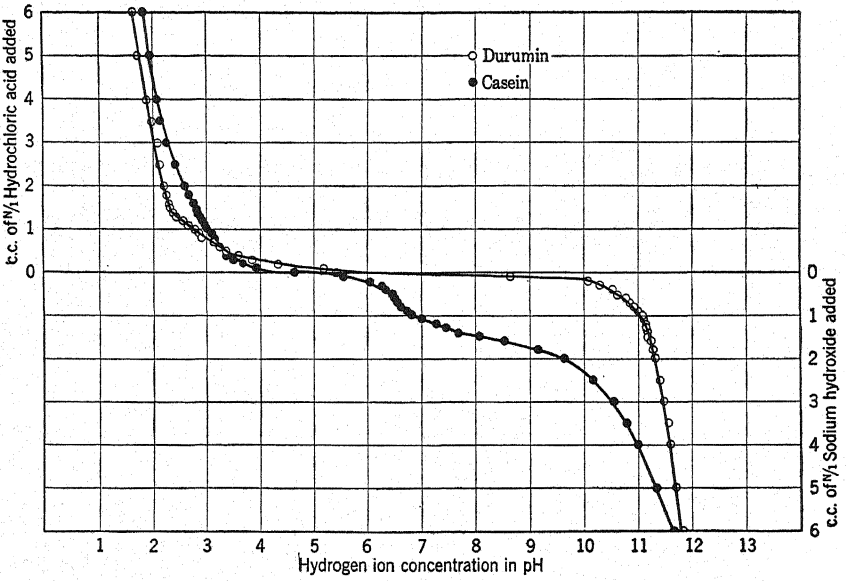


FIG. 43.—Buffer curves of durumin and casein in various concentrations of hydrochloric acid and sodium hydroxide. (Data of Hoffman and Gortner.)

bicarbonates, and phosphates are the principal buffers of biological systems still holds. Dunne[33] discusses in some detail the relation of buffer systems to the problems of plant physiology.

[33] Dunne, T. C., Plant Buffer Systems in Relation to the Absorption of Bases by Plants, *Hilgardia*, 7: (No. 5), 207–234 (1932).

CHAPTER V

OXIDATION-REDUCTION *

We shall limit this short discussion to a consideration of electronic oxidation-reduction, and attempt only to outline the general theory of such systems, together with the experimental technic involving the use of indicators and electrometric methods followed by a mention of some of the more important biological systems.[1]

The general term of oxidation-reduction is in some respects an unfortunate one. It is true that there are many reactions involving oxygen which come under this heading, for example:

$$2HCHO + O_2 \rightarrow 2HCOOH$$

but, on the other hand, there are a large number of reactions which are termed oxidations and which do not involve oxygen.

$$FeCl_2 + Cl^- \rightleftarrows FeCl_3$$

In the above reaction, the ferrous ion has been said to be oxidized to the ferric state, although no oxygen has been used. Oxidation in this case involves the loss of an electron.

$$Fe^{++} \rightleftarrows Fe^{+++} + (e)$$

In the same reaction the chlorine was reduced; it gained an electron. We can generalize this experience. If any component of a system is reduced, there must be a simultaneous and equivalent oxidation

*This chapter is contributed by Dr. H. B. Bull, Assistant Professor of Physiological Chemistry in Northwestern University; formerly on the staff of the Division of Agricultural Biochemistry, University of Minnesota.

[1] For more extended discussions of the field of oxidation-reduction the reader is referred to Clark, W. M., *et al.*, Studies on Oxidation-Reduction, I–X, U. S. Public Health Service, Hygienic Laboratory Bull. 151, 363 pp., Washington (1928); Michaelis, L., Oxidation-Reduction Potentials (translated by L. B. Flexner), 199 pp., J. B. Lippincott Company, Philadelphia and London (1930): Kolthoff, I. M., The Colorimetric and Potentiometric Determination of *p*H, 167 pp., John Wiley & Sons, New York (1931); Kolthoff, I. M., and Furman, N. H., Potentiometric Titrations, Second Edition, 482 pp., John Wiley & Sons, New York (1931); Hewitt, L. F., Oxidation-Reduction Potentials in Bacteriology and Biochemistry, Third Edition, 90 pp., London County Council, London, England (1935); Glasstone, S., and Hickling, A., Electrolytic Oxidation and Reduction: Inorganic and Organic, 420 pp., D. Van Nostrand Company, New York (1936).

of some other component, and *vice versa*. On the basis of the above example, we may formulate a general definition of an oxidation or a reduction; *an oxidation involves the loss and a reduction a gain of electrons*. According to this definition, a metal in the presence of its ions is an oxidation-reduction system.

$$Ag \underset{\text{reduction}}{\overset{\text{oxidation}}{\rightleftarrows}} Ag^{+} + (e)$$

An example with which all biologists are familiar is the hydrogen electrode. Here the hydrogen gas is the reduced and the hydrogen ions are the oxidized form.

The oxidation-reduction potential is a measure of the tendency for a substance to give up or take up electrons. Stated in a different way, it is a quantitative measure of the free energy of the reaction which is involved in the electronic transfer. Evidently in order to have a potential established, there must be an electronic transfer, although, with some reactions involving no such transfer, there are certain tricks which can sometimes be resorted to and which yield an apparent potential.

Electrode Potentials.—If an inert metal such as platinum is immersed in a reversible oxidation-reduction system, and this electrode connected to a potentiometer together with a calomel half cell in the same way as is done with the hydrogen electrode, a potential difference will be observed. It can be shown theoretically and experimentally that this potential is a function of the ratio of the oxidized to the reduced form; the more of the oxidized component, the higher the potential, and the more of the reduced form, the more negative will be the potential.

In order to indicate how the relation between the measured potential difference and the ratio of the oxidized and reduced forms is derived, the reaction

$$Fe^{++} \rightleftarrows Fe^{+++} + (e)$$

may be taken as an example. At equilibrium this reaction can be formulated as

$$\frac{[Fe^{+++}][e]}{[Fe^{++}]} = K_1 \qquad (49)$$

Now, if a platinum wire is immersed in this system, there will be a tendency for the electrons in solution to flow into the wire. On the other hand, the wire already has a concentration of electrons, and the direction of flow will be governed by these two concentrations. It can

be shown that the work in calories done in transferring an equivalent of electrons from the solution into the wire will be

$$\text{Work} = RT \log_e \frac{[e_m]}{[e_s]} \tag{50}$$

where $[e_s]$ = the concentration of electrons in the solution;
$[e_m]$ = the electronic concentration in the metal; and
R = the gas constant = 1.99 calories.

The work is equal to the quantity of electricity transferred, multiplied by the potential at which the transfer was made. We can substitute these terms in equation (50) and rearrange it to obtain

$$nEF = RT \log_e [e_m] + RT \log_e \frac{1}{[e_s]} \tag{51}$$

where E = the oxidation-reduction potential;
F = the faraday;
n = the number of equivalents; and
R = the gas constant, expressed in joules.

We now substitute equation (49) in equation (50), and since the concentration of the electrons in the metal, $[e_m]$, is a constant, it can be combined with K_1 of equation (49) to form a new constant, K_2, then

$$E = K_2 + \frac{RT}{nF} \log_e \frac{[\text{Fe}^{+++}]}{[\text{Fe}^{++}]} \tag{52}$$

When the concentration of the ferric iron equals that of the ferrous iron, we have the logarithm of 1, which is equal to zero, and then $E = K_2$. E is usually written E_h and K_2 is substituted by E_0. Then we have

$$E_h = E_0 + \frac{RT}{nF} \log_e \frac{[\text{Fe}^{+++}]}{[\text{Fe}^{++}]} \tag{53}$$

This equation can be generalized in the form

$$E_h = E_0 + \frac{RT}{nF} \log_e \frac{\text{Ox}}{\text{Red}} \tag{54}$$

At 30° C. and log to the base 10 and one electron transfer

$$E_h = E_0 + .06 \log \frac{\text{Ox}}{\text{Red}} \tag{55}$$

for a reduction involving two electrons at 30° C.

$$E_h = E_0 + .03 \log \frac{\text{Ox}}{\text{Red}} \tag{56}$$

The oxidation-reduction potential is always referred to the normal hydrogen electrode which is assumed to have zero potential. In practice, it is usually more convenient to use a calomel half cell, and correct for the potential contributed by the calomel half cell; always, however, in reference to the normal hydrogen electrode. E_0 of equation (54) has considerable significance. As pointed out above, if the concentration of the oxidant equals that of the reductant, this term becomes zero and the observed potential is equal to E_0. This is the potential which is used to compare the several oxidation - reduction systems.

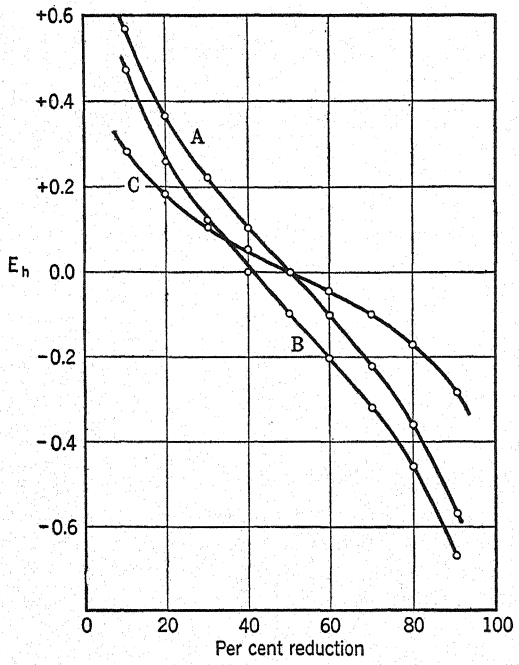


FIG. 44.—Redox potential as a function of per cent reduction. (*A*) one electron, (*B*) one electron, (*C*) two electrons.

It should be added that the oxidation-reduction potential is an *intensity* factor in the same way that temperature is an intensity factor and does not in any way indicate the reducing or oxidizing capacity of a system.

Figure 44 shows the plot of equation (54) for three hypothetical systems involving the transfer of one and two equivalents.

We see from Figure 44 that, although system *A* has a higher oxidation potential than system *B*, if system *B* contains 90 per cent of the oxidized form, it will tend to oxidize system *A*, if that system contains 90 per cent of the reduced and 10 per cent of the oxidized form.

The sign and magnitude of the oxidation-reduction potential tells us nothing concerning the relative speeds of oxidation and reduction. Thermodynamics is not capable of giving us any information on this score. Oxidation-reduction potential studies give us a negative answer. We can say definitely that one system with a lower potential can never oxidize a system with a higher potential, but we cannot say definitely that a system at a higher potential will oxidize a system of

a lower potential. The reaction may not proceed or, if it does, it may go so slowly that it is of no practical interest. Often, however, an experimental relation is observed between the speed of oxidation and the difference in potential between the two systems. Michaelis and Smythe [2] found that in a series of iron compounds the autoxidizability of the ferro compound at a given *p*H closely parallels the normal oxidation-reduction potential of the system, ferro compound-ferri compound, at the same *p*H. The more negative the potential, the greater was the autoxidizability.

EFFECT OF HYDROGEN IONS.—Hydrogen-ion concentration must usually be taken into consideration when dealing with oxidation-reduction systems because in many cases the reduced form is an anion which can accept hydrogen ions and so become inoperative in contributing to the potential. Naturally, the potential of such a system is greatly influenced by the hydrogen-ion concentration. Quinhydrone is such a system.

An oxidation-reduction system involving the formation of an anion may be expressed as

$$\mathrm{Red}^- \rightleftarrows \mathrm{Ox} + (e)$$

and the electrode equation is

$$E_h = E_0 + \frac{RT}{nF} \log_e \frac{\mathrm{Ox}}{\mathrm{Red}^-} \tag{57}$$

but the reduced form ionizes as follows

$$\mathrm{H\ Red} \rightleftarrows \mathrm{H}^+ + \mathrm{Red}^-$$

and

$$\frac{[\mathrm{H}^+][\mathrm{Red}^-]}{[\mathrm{H\ Red}]} = K \tag{58}$$

The total reduced form Red is equal to the ionized plus the unionized forms.

$$\mathrm{Red} = \mathrm{H\ Red} + \mathrm{Red}^- \tag{59}$$

Then, combining equations (58) and (59), we have

$$\frac{[\mathrm{H}^+][\mathrm{Red}^-]}{[\mathrm{Red}] - [\mathrm{Red}^-]} = K \tag{60}$$

or

$$[\mathrm{Red}^-] = \frac{[\mathrm{Red}]K}{[\mathrm{H}^+] + K} \tag{61}$$

[2] Michaelis, L., and Smythe, C. V., The Correlation Between Rate of Oxidation and Potential in Iron Systems, *J. Biol. Chem.*, 94: 329–340 (1931).

And substituting equation (61) in the electrode equation (57) we have

$$E_h = E_0 + \frac{RT}{F} \log_e \frac{\text{Ox}}{\text{Red}} \left[\frac{[\text{H}^+] + K}{K} \right] \tag{62}$$

or

$$E_h = E_0 + \frac{RT}{F} \log_e \frac{\text{Ox}}{\text{Red}} - \frac{RT}{nF} \log_e \frac{K}{[\text{H}^+] + K} \tag{63}$$

If the ratio of oxidized to reduced is equal to 1, equation (63) becomes

$$E_h = E_0 - \frac{RT}{F} \log_e \frac{K}{[\text{H}^+] + K} \tag{64}$$

If the value of K is so small in comparison with the hydrogen-ion concentration that it can be neglected, we have

$$E_h = E_0 - \frac{RT}{F} \log_e \frac{K}{[\text{H}^+]} \tag{65}$$

or

$$E_h = E_1 - \frac{RT}{F} \log_e \frac{1}{\text{H}} \tag{66}$$

And at 30° C.

$$E_h = E_1 - 0.06\, p\text{H} \tag{67}$$

and the system could be used to determine the hydrogen-ion concentration.

The above example involving the formation of an anion with the transfer of only one electron is very unusual for organic compounds and was selected because it presents the minimum of complexities. As a general rule two electrons are involved in the oxidation-reduction reaction of organic compounds, and it was thought for a number of years that these electrons had to be transferred simultaneously. Michaelis,[3] however, has pointed out that in the case of several organic systems the electrons are transferred stepwise and that a definite semiquinone compound is formed. He has established the criteria for deciding whether the electronic transfer occurs in one or two steps. Michaelis and co-workers have studied a number of systems involving stepwise transfers of electrons, and in a recent paper [4] described the case of phenanthrenequinone sulfonate.

Shaffer [5] suggests that the ability of some dyes to catalyze certain oxidation-reduction reactions is related to this property of yielding

[3] Michaelis, L., Semiquinones, the Intermediate Steps of Reversible Organic Oxidation-Reduction, *Chem. Rev.*, 16: 243–286 (1935).

[4] Michaelis, L., and Schubert, M. P., Some Problems in Two-Step Oxidation Treated for the Case of Phenanthrenequinone-sulfonate, *J. Biol. Chem.*, 119: 133–140 (1937).

[5] Shaffer, P. A., Catalysis of Ionic Oxidation-Reduction Reactions by Dyes and its Probable Mechanism, *J. Phys. Chem.*, 40: 1021–1026 (1936).

electrons stepwise. It is known, for example, that the reaction between Ti^{3+} and I_3^- is a slow one; Ti^{3+} can give up one electron but not two, while I_3^- can accept two but not one. If a small amount of a dye capable of stepwise oxidation-reduction be added, the reaction between Ti^{3+} and I_3^- is greatly accelerated. The dye has taken electrons one at a time from Ti^{3+} and given them up two at a time to I_3^-.

In addition to the formation of an anion as formulated above, the oxidized form may be a cation which is capable of accepting hydroxyl ions, or we may have the simultaneous destruction of a cation and the creation of an anion. These complications will not be dealt with in detail; suffice it to say that the oxidation-reduction potential usually varies in a highly characteristic manner with the hydrogen-ion concentration.[6] The viologens, however, described by Michaelis and Hill[7] are an exception, as their potentials are not affected by changes in hydrogen-ion concentration.

The dependence of several oxidation-reduction systems on *p*H is shown in Figure 45.

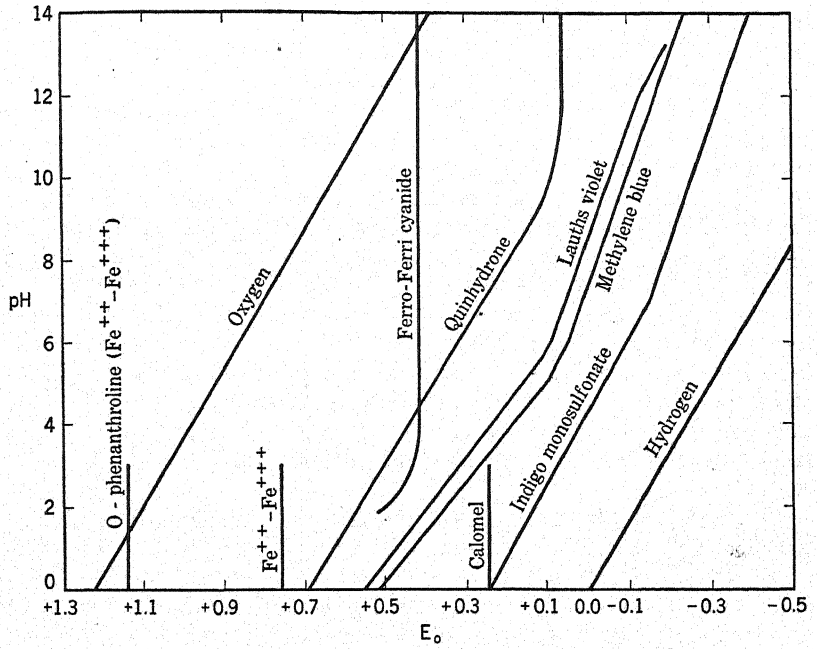


FIG. 45.—E_0 values as a function of *p*H for various redox systems.

[6] Clark, W. M., and Cohen, B., Studies on Oxidation-Reduction II., An Analysis of the Theoretical Relations between Reduction Potentials and *p*H, *U. S. Pub. Health Repts.*, 38: 666–683 (1923).

[7] Michaelis, L., and Hill, E. S., The Viologen Indicators, *J. Gen. Physiol.*, 16: 859–873 (1933).

The ionization constants of the oxidation-reduction systems are equal to the hydrogen-ion concentration at the inflection points of the curves ($p\mathrm{K} = p\mathrm{H}$). The sign in the change in slope of the curve at the inflection point indicates whether the dissociation belongs to the oxidant or to the reductant. When the change in slope $\left(-\frac{dE}{dp\mathrm{H}}\right)$ is negative, this change is due to the reductant, and when positive to the oxidant. It is also possible to evaluate the dissociation constants by a simple acid-base titration.[8]

A very fundamental analogy exists between acids and bases on one hand and oxidants and reductants on the other. For, according to Brönsted, the relation between the generalized acid A and base B is

$$\mathrm{A} \rightleftarrows \mathrm{B} + (+)$$

where $(+)$ is a proton. We have already seen how oxidation-reduction can be formulated as

$$\mathrm{Red} \rightleftarrows \mathrm{Ox} + (e)$$

where (e) is the electron. Thus we see that the creation of an acid involves the addition of the proton to the base, while the creation of the reductant involves the addition of an electron to the oxidant.

rH.—We have indicated above how the oxidation-reduction potential depends on *p*H. Clark and Cohen, in an attempt to define completely oxidation-reduction systems, invented the term *r*H, which is the logarithm of the reciprocal of the pressure of the hydrogen gas in equilibrium with the system. It has since been recommended by Clark that the use of this term be discontinued. The difficulty is that, before *r*H has any meaning, it is first necessary to know the relation between the oxidation-reduction potential and *p*H, and for most biological systems we have no such knowledge.

To summarize this theoretical discussion, we may state that the oxidation-reduction potential depends on three conditions: (1) the innate tendency of a system to take up or give off electrons, (2) the ratio of the oxidant to the reductant, and (3) the hydrogen-ion concentration.

Measurement of the Oxidation-Reduction Potential.—As with the measurement of the hydrogen-ion concentration, two methods are in general use: the indicator and the electrometric methods. For various reasons, however, indicators are less reliable in the measurement of the oxidation-reduction potential than for the measurement of *p*H. These two methods will now be considered.

[8] Hall, W. L., Preisler, P. W., and Cohen, B., Studies on Oxidation-Reduction XIV. Equilibrium Potentials of 2,6-Dibromobenzenone Indophenol-2′-Sodium Sulphonate, 2,6-Dibromobenzenone Indophenol-3′-Sodium Sulphonate, 2,6-Dichlorobenzenenone Indo-2′-Chlorophenol, and 2,6-Dimethylbenzenone Indophenol, Supplement 71 to *U. S. Pub. Health Repts.* (1928), 26 pp..

Colorimetric Method.—It is no accident that a substance which easily undergoes oxidation and reduction is usually colored. Such a substance possesses loosely held electrons which can be transferred without difficulty to another substance. The vibrations of these electrons in the molecule are responsible for the color. Clark and coworkers and others have established a series of oxidation-reduction indicators whose potentials and ionization constants are known. By observing what indicators are oxidized or reduced by the system under investigation, it is possible to assign to the system its approximate oxidation-reduction potential. The indicator method has proved useful in the study of living cells, where it has not as yet been possible to devise an electrode which would not injure the cell. Where attempts at electrode measurement have been made, the cell after a very short time apparently forms a vacuole around the electrode, and the measured potential is not that of the cell proper. Indicators are also of great value in quantitative analysis in determining the end point of an oxidation-reduction reaction. It is also possible for the dye to serve as the oxidizing agent as well as an indicator. For example, Farmer and Abt [9] use 2,6-dichlorobenzenoneindophenol to estimate the ascorbic acid content of small amounts of blood, and this indicator has come into rather general use for the titrametric estimation of ascorbic acid (vitamin C).

Table XVIII lists some of the more common indicators which, under certain circumstances, may be used to an advantage.

In general there are five considerations which determine the choice of the indicator to be used:

1. The indicator must not act as a hydrogen-ion indicator at the *p*H of the system under investigation, since an alteration in color might be due to a change in *p*H and would thus lead to confusion.

2. The normal oxidation-reduction potential of the indicator must be close to that of the system under study.

3. The indicator must possess a distinctive color, in order not to be confused with the natural color of the system.

4. The indicator should have an intense color, in order that very low concentrations may be used. A number of biological systems have a definite oxidation-reduction potential but possess only a small capacity, *i.e.*, only a small fraction of the system is effective at any given time, although potentially it may have a large reducing or oxidizing capacity. If an excess of dye is used, it may completely oxidize or reduce the active part of the biological system, and thus lead to an erroneous idea as to the actual reducing intensity of the cell. A considerable lapse of time may be necessary to determine the true reducing level of the cell.

[9] Farmer, C. J., and Abt, A. F., Determination of Reduced Ascorbic Acid in Small Amounts of Blood, *Proc. Soc. Exptl. Biol. Med.*, 34: 146–150 (1936).

TABLE XVIII

E_0 VALUES FOR OXIDATION-REDUCTION INDICATORS AT 30° C. AS A FUNCTION OF pH

Indicator	pH										
	5.0	5.4	5.8	6.2	6.6	7.0	7.4	7.8	8.2	8.6	9.0
	E_0	E_0	E_0	E_0	E_0	E_0	E_0	E_0	E_0	E_0	E_0
Phenol-*m*-sulfonate indo-2.6-dibromophenol	+0.390	+0.366	+0.342	+0.319	+0.295	+0.273	+0.251	+0.229	+0.207	+0.187	+0.168
o-Chlorophenol indophenol	.....	.....	.....	+0.288	+0.262	+0.233	+0.203	+0.170	+0.139	+0.109	+0.082
Phenol indophenol	.....	.....	.....	+0.276	+0.254	+0.227	+0.200	+0.170	+0.139	+0.110	+0.083
o-Bromophenol indophenol	.....	.....	+0.308	+0.284	+0.259	+0.230	+0.200	+0.167	+0.137	+0.105	+0.079
2.6-Dichlorophenol indophenol	+0.366	+0.339	+0.310	+0.279	+0.247	+0.217	+0.189	+0.162	+0.137	+0.113	+0.089
Phenol indo-2.6-dibromophenol	.....	.....	.....	.....	.....	+0.218	+0.190	+0.163	+0.138	+0.114	+0.090
m-Cresol indophenol	.....	.....	.....	+0.259	+0.233	+0.208	+0.185	+0.160	+0.134	+0.105	+0.076
o-Cresol indophenol	.....	.....	.....	+0.243	+0.217	+0.191	+0.168	+0.143	+0.116	+0.086	+0.057
2.6-Dichlorophenol indo-*o*-cresol	+0.335	+0.307	+0.277	+0.245	+0.212	+0.181	+0.152	+0.125	+0.099	+0.075	+0.051
Toluylene blue	+0.221	+0.196	+0.173	+0.151	+0.132	+0.115	+0.101	+0.088	+0.075	+0.063	+0.051
Thymol indophenol	.....	.....	+0.244	+0.222	+0.198	+0.174	+0.148	+0.123	+0.097	+0.069	+0.041
1-Naphthol-2-SO_3H-indo-3.5-dichlorophenol	+0.262	+0.236	+0.210	+0.181	+0.150	+0.119	+0.088	+0.060	+0.034	+0.010	−0.012
Thionine	+0.138	+0.112	+0.100	+0.087	+0.074	+0.062	+0.050	+0.037	+0.025	+0.014	−0.001
Methylene blue	+0.101	+0.077	+0.056	+0.039	+0.024	+0.011	−0.002	−0.014	−0.026	−0.038	−0.050
Indigo tetrasulfonate	+0.065	+0.041	+0.017	−0.006	−0.027	−0.046	−0.062	−0.077	−0.090	−0.102	−0.114
Indigo trisulfonate	+0.032	+0.008	−0.016	−0.039	−0.061	−0.081	−0.099	−0.114	−0.127	−0.140	−0.152
Indigo disulfonate	−0.010	−0.034	−0.057	−0.081	−0.104	−0.125	−0.143	−0.160	−0.174	−0.187	−0.199

5. The indicator must not enter into the reactions of the system under observation. It must not catalyze reactions which do not ordinarily occur. And, also, it must not have toxic properties which would injure or kill the cells. It must not be adsorbed on or combine in any way with the components of the system.

In determining the reducing intensity of cells with indicators, two methods are used, either (1) the dye is injected into the cell by micro-injection technic, or (2) the dye is allowed to diffuse into the cell. The micro-injection method cannot be used at times because some cells are too sensitive to injury, or the cells may be too small. In using the diffusion method, one has to be sure that the indicator will penetrate the cell. There seems to be a marked correlation between the reducing intensity of individual cells and of a suspension of these same cells.

Electrometric Methods.—The electrometric method should be used whenever possible. As indicated above, the use of indicators is rather severely limited. The electrometric method is much broader in its application.

The aparatus used in the measurement is surprisingly simple. The quinhydrone electrode is an oxidation-reduction system which is used so generally that it seems needless to describe it. The same set-up is employed to measure the potential of any oxidation-reduction system excepting that in some cases the reductant is oxidized by the oxygen of the air, which necessitates a closed oxidation-reduction chamber through which an inert gas, such as nitrogen, is bubbled to sweep out all oxygen in the system. In such cases it is necessary to purify the nitrogen, and this is done by passing it over heated copper filings or through alkaline pyrogallol solutions. The same technic is used in the study of anaerobic cell suspensions.

Unfortunately, a number of oxidation-reduction reactions do not yield stable, well-defined potentials, and, indeed, some reactions, such as the oxidation of an aliphatic aldehyde to an acid, do not give a potential, and the potential which one obtains in such a system is due to traces of impurities. Other reactions are sluggish and only after several hours yield a stable potential. In some cases, the reason for these difficulties is obscure. In general, the trouble may be due to one of three causes: (1) The material is irreversibly oxidized or reduced, which is often due to the oxidant or reductant being irreversibly altered. (2) The oxidation-reduction is not of an ionic nature and cannot be expected to yield a potential. Perhaps the reason for the delay in the attainment of equilibrium in the case of the sluggish systems is that part of the reaction is not ionic but involves some internal change or rearrangement within the molecule. Ionic reactions, as a rule, proceed with extreme rapidity. (3) Mention has already been made of the suggestion of Shaffer that the difficulty often lies in the

reaction between a compound involving a change of one electron with a system involving a change of two electrons.

Conant [10] and others have studied the irreversible oxidation-reduction of organic compounds and have developed methods for determining what they term the apparent potentials. This is done by choosing some easily reversible systems with the oxidant and reductant in equivalent amounts and using them in conjunction with an inert electrode. The substance under investigation is added, and the potential, which is due to the reversible system, is observed. If a 20 to 30 per cent reduction of the reversible system has been accomplished in 30 minutes, the apparent reducing potential is said to be that of the reversible system.

Other methods have been used. Some substances which do not of their own accord exhibit true potentials can be titrated with a reversibly reducible oxidant. For example, potassium ferricyanide has been used to titrate the reduced form of ascorbic acid. The ascorbic acid is oxidized and the ferricyanide is reduced. The observed potential is due to the ferri-ferrocyanide system, but if sufficient time has been allowed for attainment of equilibrium, this potential must be equal to that of the ascorbic acid system and will continue to be so until all the reduced ascorbic acid has been oxidized.

Some systems are not reversibly reduced until their molecules are activated with an enzyme or, in some cases, by a dyestuff. The reduction of fumaric to succinic acid is reversible only in the presence of a succinic dehydrogenase and oxidation-reduction indicators.

The important sulfhydryl systems have not, as yet, yielded to theoretical treatment. The potential of the cystine-cysteine, as well as the oxidized-reduced glutathione, is dependent on the concentration of the reductant but not on that of the oxidant.

Biological Systems.—A number of systems of biological interest have been studied and, to some extent, characterized. These include systems containing sulfhydryl, hemoglobin, cytochrome, respiratory pigment, ascorbic acid, hermidine and echinochrome, pyrocyanine, dilauric acid, various sugar systems, succinate-fumarate, adrenaline, oxytocic hormone of pituitary gland, as well as tissue and cell suspensions. All together, about 15 biological pigments have been found to constitute oxidation-reduction systems, and respiratory functions have been attributed to some of these pigments. This represents a definite and worthwhile achievement; however, the biological systems which yield reversible potentials, and are thus capable of an exact thermodynamic treatment, are relatively few, and it appeared a few years ago as if the study of oxidation-reduction must soon be completed with the characterization of these systems. Later work has shown, however,

[10] Conant, J. B., The Electrochemical Formulation of the Irreversible Reduction and Oxidation of Organic Compounds, *Chem. Rev.*, 3: 1–40 (1926).

that there is a relation between the reducing potential of cell suspensions and of physiological systems in general and their physiological condition. This realization has added a new interest to the study of oxidation-reduction potentials. The theoretical interpretation of these systems would be extremely difficult, if not impossible. *The derivation of the oxidation-reduction equations rests on the assumption that the system is in equilibrium. It is questionable that any living cell can be said to be in a state of true equilibrium,* and the best that can be achieved is a steady state. Under these circumstances, it is not valid to apply the above equations. The field must be examined experimentally, and the reducing intensity related to known physiological conditions. This method of approach has already proceeded a considerable distance and is proving of especial value in bacteriology.[11]

The assistance which oxidation-reduction potential measurements can yield in the characterizing of a physiological state is strikingly illustrated by the study of Rosner.[12] He studied the oxidation-reduction potentials of lenses of rats before and after the feeding of galactose. It is well known that galactose feeding results in a high incidence of cataract in rats. He found for young rats (6 weeks old, *ca.* 45 grams) average potentials of +19 mv. (range 0 to +39 mv.) for the potential of the cortex and +40 mv. (range −13 to +117 mv.) for the potential of the nucleus, whereas after galactose feeding the corresponding potentials were: cortex, +54 mv. (range +7 to +79 mv.); and nucleus, +150 mv. (range +136 to +160 mv.). For older rats (1 year *ca.* 190 grams) before galactose feeding the potentials were: cortex, +7 mv. (range −18 to +24 mv.); and nucleus, +132 mv. (range +119 to +150 mv.); after galactose feeding: cortex, +42 mv. (range +32 to +60 mv.); and nucleus, +153 mv. (range +147 to +158 mv.).

[11] Hewitt, L. F., *loc. cit.*

[12] Rosner, L., Studies on Oxidative Mechanisms in the Lens, Ph.D. thesis submitted to the Graduate School of Northwestern University, July, 1937.

CHAPTER VI

ELECTROKINETIC PHENOMENA OF COLLOID SYSTEMS

Freundlich [1,2] has designated certain electrical properties of colloid systems by the term electrokinetic phenomena in order to distinguish them from a similar, although not identical, electrical phenomenon known as the thermodynamic potential which exists at interfaces. As Freundlich points out, the electrokinetic phenomena are very closely correlated with many physical properties of colloid systems. As we shall see later, *the phenomena of adsorption, interfacial tension, mutual precipitation, flocculation, colloid stability, and the behavior of a colloid system under the influence of an electric current are all related to the so-called electrokinetic phenomena.*

Abramson [3] has recently discussed in detail the historical background, the derivation of the various mathematical equations, and the application of electrokinetic technic to a great variety of problems. In view of the ready availability of this excellent monograph it would be superfluous to repeat much of the historical background and enter into the involved mathematical derivations. The reader is accordingly referred to this monograph for such essential details. Suffice it to say that the first observation was made by Reuss, in 1808. He prepared a voltaic pile consisting of 92 silver rubles and 92 zinc plates separated by cloths moistened with salt solution. This battery was attached by wires to a U-tube 0.25 inch in diameter containing powdered quartz in the bottom of the U, the quartz being covered with water. He noted that on applying the current the water level rose approximately 9 inches above the quartz layer on the side in which the negative electrode was inserted and fell correspondingly on the side attached to the positive pole. This observation of Reuss was only eight years after the experiment of Nicholson and Carlyle (1800), who demonstrated the decomposition of water by the galvanic current, and grew out of a repetition of that experiment. Reuss also demonstrated that a block of moist clay could act as a diaphragm.

[1] Freundlich, H., Colloid and Capillary Chemistry, translated by H. S. Hatfield, E. P. Dutton and Company, New York (1926).

[2] Freundlich, H., New Conceptions in Colloidal Chemistry, E. P. Dutton and Company, New York (1927).

[3] Abramson, H. A., Electrokinetic Phenomena, Am. Chem. Soc. Monograph 66, 331 pp., Chemical Catalog Company, New York (1934); *cf.* also Prausnitz, P. H., and Reitstötter, J., Elektrophorese, Elektro-osmose, Elektrodialyse in Flüssigkeiten, 307 pp., Theodor Steinkopff, Dresden and Leipzig (1931).

In 1816, Porret further studied the phenomenon using both sand membranes and animal (bladder) membranes. He found that water migrated through bladder membrane toward the negative pole. He also coated filter paper with egg white, coagulated the egg white by heat, and then showed a migration with this membrane similar to that which had occurred with the bladder membrane, and in the same paper suggested that minute electric currents may have a great influence in regulating the flow of water through minute pores in living tissues, adding, "Is not this electrofiltration jointly with electrochemical action in constant operation in the minute vessels and pores of the animal system?"

Becquerel, about 1830, observed transport of clay particles in an electric field, and in 1852 Wiedemann showed that the amount of liquid flowing through a porous diaphragm was proportional to the e.m.f. which had been applied. The next major contribution was that of Quincke,[4] who extended the observation of Wiedemann and measured the rate of streaming and the direction of streaming for membranes of various materials. He found that the direction of streaming was determined by the material of the membrane and might be toward either the positive or the negative pole. He also observed that the rate of streaming was greatly influenced by the nature of the material. He furthermore showed that, since an e.m.f. would produce streaming, then conversely streaming would produce an e.m.f. He next turned to the study of suspended particles and showed that most suspended particles were charged negatively in water and positively in turpentine; and lastly, to account for the electrical effects, he developed a theory of a charge of one sign on the wall of the capillary and of an opposite charge in the liquid bathing the wall of the capillary. This is apparently the first suggestion of an electric double layer.

Here the theory remained until the epoch-making contributions of Helmholtz[5] in 1879. Helmholtz developed the theory of the double layer from the theory of a condenser. If Q is the charge and V_1 and V_2 are the potentials on two plates, and K is a constant, then $V_1 - V_2$ is the difference in potential and

$$Q = K(V_1 - V_2) \tag{68}$$

the capacity (C) at constant Q is

$$C = \frac{SK_1}{4\pi\delta} \tag{69}$$

[4] Quincke, G., Über die Fortführung materieller Theilchen durch strömende Elektricität, *Pogg. Ann.*, 113: 513–598 (1861); *cf.* also *Pogg. Ann.*, 107: 1–47 (1859).

[5] Helmholtz, H., Studien über elektrische Grenzschichten, *Wied. Ann.*, 7: 337–381 (1879); *Wiss. Abhandl.*, 1: 855–898 (1882).

where S = surface area;
δ = distance between plates;
K_1 = a constant.

It will be noted that the capacity is directly proportional to the area and inversely proportional to the distance between the plates. Accordingly the capacity can be increased by increasing the area or by decreasing the distance which separates the opposite electrical charges. A small thickness and a great difference in potential give a large ratio of $(V_1 - V_2)/\delta$, which is a measure of the mean electrical intensity of the field. Similarly the energy of the field (W) can be defined as

$$W = \frac{KS(V_1 - V_2)^2}{8\pi\delta} \tag{70}$$

Again the energy is directly proportional to the charge and inversely proportional to the distance.

The above elementary treatment of condensers would not have been introduced at this point except for the fact that Helmholtz in his derivations made certain assumptions, and these assumptions have until recently been overlooked or have been considered as fixed quantities. Thus he states that for simplifying the problem of a varying charge or potential, δ (the distance between the plates) may be considered as fixed and equal to the diameter of one molecule. This statement was later interpreted as Helmholtz stating that δ was a fixed quantity and equal to one molecular diameter. It remained for Gouy,[6] in 1910, to point out that δ varies and in fact may vary enormously. Gouy calculates that at the surface of 0.10 N NaCl solution δ approximates 0.96 mμ, for a 0.001 N NaCl solution δ approximates 9.6 mμ, and for pure water δ approximates 1010 mμ, assuming that the dielectric constant of 80 for water exists unchanged in the three solutions.

Practically all the existing textbooks in colloid chemistry contain the statement that electrolytes added to the colloid system reduce the electric charge and that, when the charge is reduced to approximately zero, the system becomes unstable and the colloid particles flocculate. This statement is based upon the theory of a rigid Helmholtz double layer where δ is a fixed quantity. It is obvious from a consideration of the Gouy diffuse layer that the energy of the electric field can be altered by a change in either the density of the charge or in the distances which separate charges of opposite sign, and, as we shall see later, the addition of electrolytes more often than not causes an *increase* in charge but causes δ to decrease greatly so that finally the double layer collapses. At the point of collapse the colloid floc-

[6] Gouy, M., Sur la constitution de la charge électrique à la surface d'un électrolyte, *J. phys.*, (4)9: 457–468 (1910).

culates. A further addition of electrolytes may cause the double layer to reverse, in which case the colloid is again stabilized but possesses an electrokinetic potential of the opposite sign. Fig. 46 illustrates diagrammatically a hypothetical surface (negatively charged) and three hypothetical distributions of the corresponding positive charges. In *A* the positive charges are all close to the corresponding negative charges on the surface and the plane *xy*, which separates the movable and immovable layers, lies just outside of the equivalent number of positive charges. The fall of potential accordingly is entirely within the double layer corresponding to an electrokinetic potential of zero, *i.e.*, there is no difference in ionic charge within and without the double layer. In *B* we have a somewhat diffuse Gouy layer, some of the positive charges lying outside of the plane *xy*. Accordingly there is a difference in ionic charge within and without the double layer, and

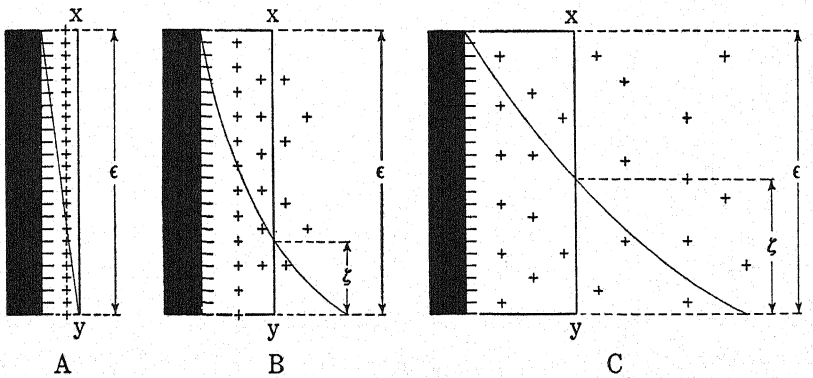


FIG. 46.—A diagrammatic representation of the relationships between the ε-potential and the ζ-potential at constant charge density, but with a varying thickness of the double layer due to a different ionic distribution.

the fall of potential, though predominantly within the immovable layer, is partly outside of the immovable layer and gives rise to an appreciable electrokinetic potential. In *C* the positive charges are equally distributed within and without the immovable layer. The thickness (δ) of the Helmholtz layer is increased and the electrokinetic potential is likewise increased, and at the same time there is a lower charge density within the double layer. It should be noted that in all three of these diagrams the thermodynamic or ϵ-potential remains constant.

We have already noted that an electrical charge on a colloidal micelle may arise from several causes, such as the *direct ionization* of the material comprising the micelle, the *capture of an ion* by a micelle (adsorption), or *electrification by contact*. Michaelis [7] classifies the

[7] Michaelis, L., The Effects of Ions in Colloidal Systems, Williams and Wilkins Company, Baltimore (1925).

causes as due to (1) the forces of residual valences which cause oriented adsorption, (2) the forces of dissociation which cause exchange adsorption, and (3) the spontaneous distribution of ions at a free surface which comes into play in those instances where the electric double layer is set up at the boundary of substances which are incapable of dissociation and which do not react chemically.

In the first instance, silver bromide crystals, for example, contain in their crystal faces both silver and bromine atoms, the charges of which are not completely neutralized. Accordingly, if there is an excess of bromine ions in the surrounding liquid, they will be attracted to the vicinity of the unsaturated silver atoms in the crystal face; if there is an excess of silver ions in the surrounding solution, they will be attracted to the unsaturated bromine atoms in the crystal face. The result has already been shown diagrammatically in Figure 5. In the event that the bromine ions are in excess, the colloid micelle will be negatively charged, whereas it will be positively charged if the silver ions are in excess. The electric double layer is, therefore, reversed as we pass the isoelectric point. No difference in potential in the double layer will exist at the isoelectric point.

It is easy to picture how dissociation brings about an electric double layer. The dissociation of a ferric hydroxide sol would result in the negative hydroxyl ion being given off into the solution with a corresponding residual positive charge remaining on the micelle. The negative charge cannot be separated by any great distance from the residual positive charge. Accordingly the double layer is set up.

The third class of substances noted by Michaelis comprises such substances as cellulose, collodion, air bubbles, colloidal carbon, hydrocarbons, etc., where there is probably no great tendency to dissociate and a relatively slight tendency toward oriented adsorption. In this instance Michaelis assumes a selective adsorption of either hydrogen or hydroxyl ions from the aqueous phase (dispersions medium). Assuming that OH^- is more capillary active than H^+, certain of the hydroxyl ions of water would enter more closely to the surface of the disperse phase, so that there would be near the surface of the disperse phase a greater concentration of hydroxyl ions than of hydrogen ions. Accordingly, a double layer would be set up, the micelle acquiring a negative charge.

Helmholtz (*loc. cit.*), Perrin,[8] Lamb,[9] and Smoluchowski[10] have expressed the theory of electroendosmosis in mathematical form. In

[8] Perrin, J., Mécanisme de l'électrisation de contact et solutions colloidales, *J. chim. phys.*, 2: 601–651 (1904); 3: 50–110 (1905).

[9] Lamb, H., On the Theory of Electric Endosmosis and Other Allied Phenomena, *Brit. Assoc. Repts.*, 495–510 (1887)

[10] von Smoluchowski, M., Contribution à la théorie de l'endosmose électrique et de quelques phénomènes corrélatifs, *Bull. Acad. Sci. Cracovie*, 182–199 (1903).

the following discussion we will make use of the mathematical treatment as presented by Briggs.[11]

Let us assume the existence of an electrical double layer on the wall of a capillary, one part of the electrical double layer being fixed to the solid phase, the other portion, located at distance (δ) from the opposite charge, being in the liquid phase and free to move with the liquid. If, now, a potential difference is applied to the ends of the capillary, one charge tends to move toward the anode, the other toward the cathode, but since the charge on the solid is fixed, the charged liquid layer will move along the surface of the capillary at such a rate that the frictional forces (R) will be at equilibrium with the electrical forces (F). The electrical force (F), acting on a unit area of surface, is equal to the product of the charge (e) on the double layer per unit of surface and the potential difference (E) which is applied to a unit length.

$$F = eE \tag{71}$$

At equilibrium this must be equal to the frictional force which must be overcome or balanced. The frictional force (R) is related to the coefficient of viscosity of the liquid and to the decrease in velocity of the flow of the liquid in a direction perpendicular to the wall of the capillary, *i.e.*, the average velocity of the liquid layer between the point of maximum velocity (u) and the point where no flow takes place, the distance between these two points being designated as δ. The frictional force can therefore be expressed as

$$R = \frac{\eta u}{\delta} \tag{72}$$

u may be evaluated by measuring the volume (V) of liquid flowing in a given time through a known area of cross section of the tube, according to the equation

$$V = \pi r^2 u \tag{73}$$

accordingly,

$$R = \frac{\eta V}{\delta \pi r^2} \tag{74}$$

and from equation (71),

$$eE = \frac{\eta V}{\delta \pi r^2} \tag{75}$$

On the assumption that the properties of the double layer are those of two plates of a flat condenser, we can state that the capacity (C) of the double layer is directly proportional to the quantity (Q) of elec-

[11] Briggs, D. R., The Determination of the ζ-Potential on Cellulose—A Method, *J. Phys. Chem.*, 32: 641–675 (1928).

tricity which it holds and inversely proportional to the potential difference (ζ) across the plates,

$$C = \frac{Q}{\zeta} \tag{76}$$

The capacity is likewise proportional to the dielectric constant (ϵ) of the material separating the plates, to the area (A) of the plates, and inversely proportional to their distance apart (δ) and to a constant, 4π. Accordingly,

$$C = \frac{\epsilon A}{4\pi\delta} \tag{77}$$

therefore,

$$\frac{Q}{\zeta} = \frac{\epsilon A}{4\pi\delta} \tag{78}$$

and

$$\zeta = \frac{4\pi\delta Q}{\epsilon A} \tag{79}$$

Substituting e, the charge per unit area, for the quantity $\frac{Q}{A}$, we have

$$\zeta = \frac{4\pi\delta e}{\epsilon} \quad \text{or} \quad \pi\delta = \frac{\zeta\epsilon}{4e} \tag{80}$$

Again substituting, we have

$$eE = \frac{\eta V 4e}{\zeta\epsilon r^2} \tag{81}$$

Accordingly,

$$V = \frac{r^2\zeta E\epsilon}{4\eta} \tag{82}$$

and

$$\zeta = \frac{4V\eta}{r^2 E\epsilon} \tag{83}$$

However, in a diaphragm the πr^2 of a capillary tube must be replaced by the area of cross section (q), r^2 equaling $\frac{q}{\pi}$. Accordingly,

$$V = \frac{q\zeta E\epsilon}{4\pi\eta} \tag{84}$$

and

$$\zeta = \frac{4\pi V\eta}{qE\epsilon} \tag{85}$$

E is equal to H, the e.m.f. applied across the diaphragm, divided by l, the distance between the ends of the diaphragm. Likewise, H, the applied e.m.f., is equal to i, the current, multiplied by w, the resistance, where w is equal to the distance, l, between the ends of the

diaphragm, divided by the product of q, the cross section area, and κ, the specific conductivity of the liquid. Therefore,

$$V = \frac{\zeta i \epsilon}{4\pi\eta\kappa} \tag{86}$$

and

$$\zeta = \frac{4\pi\eta\kappa V}{i\epsilon} \tag{87}$$

This equation states that where the electric current (i) is kept constant, the volume of liquid (V) which will flow in a given time through a diaphragm is directly proportional to the ζ-potential across the interface, to the dielectric constant (ϵ) of the liquid, and inversely proportional to the viscosity (η), and the specific conductivity of the liquid (κ) and is independent of the area of cross section or the length of the capillary.

The derivation of this equation has been given at length because methods based upon this equation have been of great service in colloid studies involving biochemical materials. Briggs has discussed the various assumptions upon which this equation is based. He points out that the equation is probably not strictly accurate in that it assumes a dielectric constant of 80 for water in the immediate vicinity of the colloid micelle, whereas in all probability the compressed water layer on the surface of the micelle has a lower dielectric constant. Likewise, in the above derivation, two distances (δ) have been evaluated as equal, while as a matter of fact they are probably not equal. It is likewise certain that δ the distance between the charges in the Helmholtz double layer is not a fixed quantity but varies widely.

Briggs likewise points out that the specific electrical conductivity (κ) is not the specific electrical conductivity of the liquid which is passing through the pores of the diaphragm but rather is the specific conductivity of the liquid in the diaphragm. Accordingly equation (87) should be rewritten by substituting κ_s, the specific conductivity of the liquid within the diaphragm, for the κ, the specific conductivity of the pure liquid.

Because of the uncertainty regarding the value of the dielectric constant within the double layer and also the uncertainty with regard to the thickness of the double layer, Bull and Gortner[12] introduced a new term, qd, into electrokinetics where q is the density of charge per square centimeter of the double layer and d is the thickness of the double layer. The product, qd (distance $\times$ charge), is symbolized as δe in equation (80); it may be thought of as the electric moment

[12] Bull, H. B., and Gortner, R. A., Electrokinetic Potentials. XI. Effect of Sodium Soaps on the Electric Moment of the Double Layer at an Aqueous-Cellulose Interface, *Physics*, 2: 21–32 (1932).

of the double layer and can be evaluated by making use of quantities which can be measured experimentally. Equations involving δe will be cited later under the discussion of the streaming potential.

THE DISTINCTION BETWEEN THE ELECTROKINETIC AND THE THERMODYNAMIC POTENTIALS.—As noted in the opening paragraph of this chapter, the electrokinetic potential is distinct from the Nernst thermodynamic potential or the boundary potential which exists across interfaces. We have already noted in the consideration of hydrogen-ion concentration that a difference in potential exists at the boundary of two solutions which differ in concentration. This boundary potential can be stated as

$$\epsilon_b = -\frac{RT}{F} \log_e \frac{C_1}{C_2} \tag{88}$$

where ϵ_b = the boundary potential;
R = the gas constant;
T = absolute temperature;
F = the faraday;
C_1 and C_2 = the respective concentrations of ions in the two solutions.

The importance of this equation in biochemical problems will be evident when we consider the Donnan equilibrium (*vide infra*).

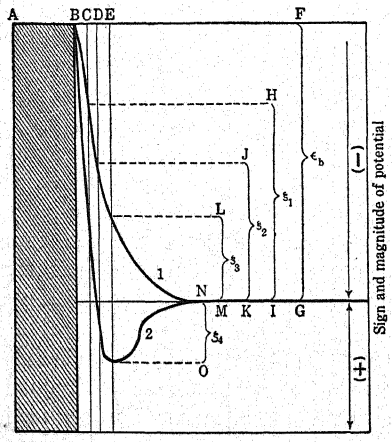


FIG. 47.—A diagrammatic representation of the possible relationship between the thermodynamic or Nernst potential and the electrokinetic or ζ-potential (for explanation see text).

Figure 47 illustrates in a diagrammatic way the relationships which may exist between ζ, the electrokinetic potential, and ϵ_b, the thermodynamic or Nernst potential, and shows how it is possible to conceive of the electrokinetic potential varying both in magnitude and in sign, while the thermodynamic potential remains constant.

Let the cross-hatched section between the lines A and B at the left-hand of Fig. 47 represent a colloid gel in equilibrium with a liquid phase. The thermodynamic potential ϵ_b would be the potential which was measured from some point on the interior of the gel to some point well within the interior of the surrounding medium. The magnitude of this potential is

represented on the diagram by the line *FG*. The electrokinetic potential, however, has already been defined as the potential which exists between the movable and immovable liquid layers at the interface. In other words, the interface which gives rise to the potential ϵ_b is the interface of gel-liquid. The interface which gives rise to ζ is the interface between the immovable liquid film adhering to the surface of the gel and the body of the liquid. Curve 1, Fig. 47, represents the hypothetical fall in electrokinetic potential from the surface of the gel into the body of the liquid. Curve 2 represents a second hypothetical fall of the electrokinetic potential. As we shall see later when we discuss the effect of ions on colloid systems, curves similar to both 1 and 2 have been demonstrated experimentally.

With a gradient of electrokinetic potential, such as is postulated in curve 1, it should be possible to have values of ζ which vary in magnitude from ζ_1 to ζ_2 to ζ_3, depending on the thickness of the immovable, adsorbed water layer adhering to the surface of the gel, assuming that the ion concentration in the immovable, adsorbed water layer varies with the thickness of the bound-water film, *i.e.*, that ions and water molecules are not necessarily adsorbed in the same ratio. We shall see when we discuss the solvation of lyophilic colloids that the thickness of the water film adsorbed to the surface of the colloid particle may vary within wide limits. As a matter of fact, we have already noted this variation, for the viscosity curves shown in Figs. 13, 14, 34, and 35 represent in reality changes in the thickness of the water film associated with the disperse phase. Accordingly if the hypothetical water film in Fig. 47 has a thickness *BC*, the boundary between the movable and immovable phases would occur at *C*, and ζ_1 would represent the magnitude of the electrokinetic potential. If, however, the water film had a thickness *BD*, the movable boundary would be shifted to *D* and the magnitude of ζ_2 would measure the electrokinetic potential. If the water film were further extended to *E*, so that *E* became the boundary between the movable and immovable portions, the electrokinetic potential would be equivalent to ζ_3. Assuming the potential gradient to fall to curve 2 and assuming line *E* to represent the boundary between the movable and the immovable layers, the electrokinetic potential curve would cut this line at point 0, and the electrokinetic potential produced would be equivalent to ζ_4. In this case, however, we have reversed the sign of the electrokinetic potential from minus to plus, *i.e.*, we have passed the isoelectric point and the charge on the gel has been reversed.

Throughout all these changes, both in the magnitude and sign of the ζ-potential, we have assumed little or no change in the thermodynamic potential ϵ_b. Probably some change does take place in the potential ϵ_b. The change which takes place, however, is according to

equation (88) directly proportional to the logarithm of the quotient of the ion concentrations in the two phases.

The electrokinetic potential, on the other hand, does not vary directly with the differences in ion concentrations across the entire interfacial region (as measured by the usual physicochemical technic within the gel and the body of the liquid) but rather with the ion concentrations in the *thin film* which is adsorbed upon the micelles and the ion concentrations in the body of the liquid. In many instances the first unit addition of electrolyte to the body of the liquid may cause a very great change in the electrokinetic potential (probably due to the fact that either the anions or the cations of the added electrolyte are nearly all concentrated in the adsorbed immovable film), whereas a second unit addition of electrolyte to the body of the liquid may cause little or no change in ζ. Figure 48 [13] shows how the ζ-potential may vary in an unpredictable manner independently of the thermodynamic potential.

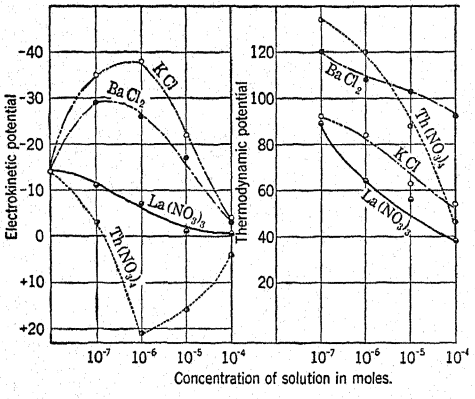


FIG. 48.—Showing that the ζ-potential and thermodynamic or Nernst potential may vary independently of each other. (Data of Freundlich and Ettisch for a glass-water interface.) Note that change in the Nernst potential is approximately proportional to the concentration, whereas there is no predictable relation between the change in the electrokinetic potential and concentration.

It is admitted that Fig. 47 is a hypothetical diagram. It is believed, however, that this diagram and the assumptions which have been made afford a reasonable explanation for many of the reactions characteristic of colloid systems.

Loeb [14] based most of his argument that protein reactions were stoichiometrical reactions on studies involving the determination of the thermodynamic potential ϵ_b at phase boundaries between proteins and the liquid with which the protein micelles were in contact. It is not surprising that Loeb reached the conclusions that the boundary

[13] Freundlich, H., and Ettisch, G., Das elektrokinetische und das thermodynamische Potential. *Z. physik. Chem.*, 116: 401–419 (1925).

[14] Loeb, J., Proteins and the Theory of Colloidal Behavior, McGraw-Hill Book Company, New York (1922).

potential varied in a quantitative manner with the ion concentration of the solutions.

As Hill [15] points out, this conclusion was an inevitable result of the manner in which the determinations were made. We shall have occasion later to discuss certain of these problems. It is only necessary at this time to point out again that, *whereas the electrokinetic potential is related to the thermodynamic potential, it may vary independently of that potential.*

Various methods have been proposed for the determination of the sign and magnitude of the electrical charge on colloidal micelles or surfaces. The methods which have been proposed may be classified as *cataphoresis, electroendosmosis, streaming potential,* and *sedimentation potential.* All these methods are interrelated and should be capable of yielding similar values.

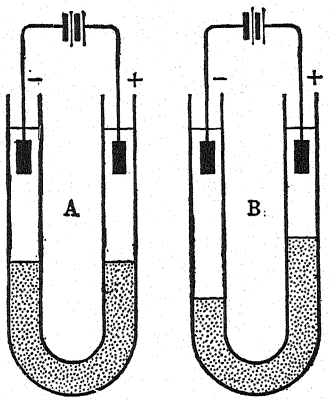


FIG. 49.—A diagrammatic representation of cataphoresis.

(*a*) CATAPHORESIS.—*Cataphoresis may be defined as the migration of the colloidal micelle through the dispersions medium, due to an imposed e.m.f.* Fig. 49 shows diagrammatically a U-tube in which a colloid sol has been layered underneath a buffer solution having the same hydrogen-ion concentration. Electrodes have been inserted in the buffer solution and connected to a source of direct current. Tube *A* shows the position of the boundary between the sol and the buffer solution before any electric current has passed. Tube *B* shows diagrammatically the displacement of the boundaries, due to the passage of the electric current, on the assumption that the sol was negatively charged. It will be noted that the colloidal micelles have migrated toward the anode in the same manner as they would have migrated if they behaved as ions. Various forms of apparatus have been devised for studying not only the direction of migration but also the rate of migration. The direction of migration indicates the sign of the charge on the colloid micelle, the colloid micelle migrating through the liquid toward the pole of the opposite sign. *The velocity of migration is proportional to the electrokinetic potential existing across the Helmholtz double layer.*

[15] Hill, A. V., The Potential Difference Occurring in a Donnan Equilibrium and the Theory of Colloidal Behavior, *Proc. Roy. Soc.* (*London*), (A), 102: 705–710 (1923).

The equations which can be applied in cataphoresis studies are

$$v = \frac{\zeta E \epsilon}{4\pi\eta} \tag{89}$$

or

$$\zeta = \frac{4\pi v \eta}{E\epsilon} \tag{90}$$

where v = velocity of migration;
E = the applied e.m.f. per unit length between electrodes;
ϵ = the dielectric constant;
η = the viscosity.

Many papers have been published dealing with the technic of the measurement of the sign and magnitude of the electrokinetic potential on suspended particles. It is practicable to note only a few of these papers in the present connection, the papers which have been selected being chosen either because of some novel form of apparatus which was used or because the papers present results which it will be desirable to refer to later in other connections.

Mattson,[16] Northrop,[17] Kunitz,[18] Northrop and Kunitz,[19] Szent-Györgyi,[20] Bull,[21] and Abramson, Moyer, and Voet [22] have described apparatus suitable for measuring cataphoretic velocity with small quantities of materials, and of microscopic particles. In these micro cells precautions must be taken to make the measurement of cataphoretic velocity at the proper depth in the cell, since there is a varying velocity of migration of the particle in all portions of the cell. This variation in migration velocity is due to the fact that in addition to the cataphoretic migration of the particle toward the pole of the opposite electric sign there is an electroendosmotic streaming (*vide infra*) of the liquid along the walls of the capillary and this liquid returns through the center of the capillary, thus producing a retarda-

[16] Mattson, S. E., Ein Mikroüberführungsapparat, *Kolloidchem. Beihefte*, 14: 309–312 (1922).

[17] Northrop, J. H., The Stability of Bacterial Suspensions. I. A Convenient Cell for Microscopic Cataphoresis Experiments, *J. Gen. Physiol.*, 4: 629–633 (1921–22).

[18] Kunitz, M., A Cell for the Measurement of Cataphoresis of Ultramicroscopic Particles, *J. Gen. Physiol.*, 6: 413–416 (1923–24).

[19] Northrop, J. H., and Kunitz, M., An Improved Type of Microscopic Electrocataphoresis Cell, *J. Gen. Physiol.*, 7: 729–730 (1924–25).

[20] Szent-Györgyi, A. v., Ein Kataphorese-Apparat fur kleine Substanzmengen, *Biochem. Z.*, 139: 74–76 (1923).

[21] Bull, H. B., Electrokinetics XIV. A Critical Comparison of Electrophoresis, Streaming Potential, and Electroosmosis, *J. Phys. Chem.*, 39: 577–583 (1935).

[22] Abramson, H. A., Moyer, L. S., and Voet, A., A Vertical Microelectrophoresis Cell with Non-Polarizable Electrodes, *J. Am. Chem. Soc.*, 58: 2362–2364 (1936).

tion and sometimes an actual reversal in sign of the direction of migration of the particle close to the wall, and on the other hand intensifies the rate of migration in the center of the capillary. There are only two levels at which true cataphoretic velocity is observed. In a flat cell these two levels lie at 21 per cent of the distance from the top or from the bottom of the cell. For a microcylindrical cell the levels lie at 14 per cent of the diameter of the capillary. Figure 50 shows a cataphoretic velocity curve for particles in a flat cell. Abramson's monograph should be referred to for a discussion of the theory of this behavior. Detailed directions for setting up the apparatus for making the measurements with a micro cell have been described by Moyer.[23]

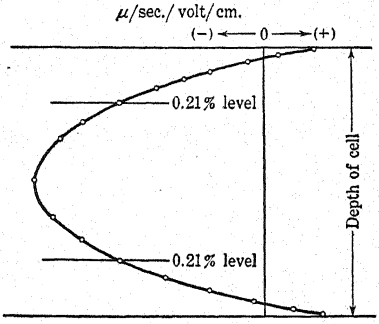


FIG. 50.—Showing cataphoretic-velocity curve for particles at different levels in a flat microcataphoretic cell. Note that correct velocity values are obtained only at the 0.21 per cent and 0.79 per cent levels.

In addition to the discussion in Abramson's monograph the reader may well consult the papers by Kruyt and Arkel,[24] and Kruyt[25] for precautions which must be observed in measurements of cataphoresis in macro cells. Svedberg and Tiselius,[26] and Scott and Svedberg[27] have studied the cataphoresis of egg albumin, using very novel technic. Figure 51 shows the cataphoresis tube which was used by Scott and Svedberg. The protein solution is inserted from reservoir *C* through stopcock *B* and is layered underneath a buffer solution, as shown in *A*. D represents a connecting tube filled with buffer solution and dipping by means of a siphon into a saturated solution of zinc sulfate. The electrodes at *E* are zinc rods. By using a zinc-zinc sulfate electrode, polarization is prevented. Similarly non-polarizable electrodes of copper-copper chloride, and of silver-sodium chloride can be used, or one can use calomel electrodes dipping into a saturated solution of potassium chloride.

[23] Moyer, L. S., A Suggested Standard Method for the Investigation of Electrophoresis, *J. Bact.*, 31: 531–546 (1936).

[24] Kruyt, H. R., and van Arkel, A. E., Eine Methode zur ultramikroskopischen Messung der kataphoretischen Geschwindigkeit, *Kolloid* Z., 32: 91–95 (1923).

[25] Kruyt, H. R., Die Methoden zur Bestimmung der Ladungsgrösse kolloider Teilchen, *Kolloid Z.*, 37: 358–365 (1925).

[26] Svedberg, The, and Tiselius, A., A New Method for Determination of the Mobility of Proteins, *J. Am. Chem. Soc.*, 48: 2272–2278 (1926).

[27] Scott, N. D., and Svedberg, The, Measurements of the Mobility of Egg Albumin at Different Acidities, *J. Am. Chem. Soc.*, 46: 2700–2707 (1924).

For exact quantitative work connection must be made through a reversible electrode; otherwise the buffer solution in the upper portion of the U-tube, as shown in Fig. 49, changes in hydrogen-ion concentration, and the diffusion of the acid or alkali formed by electrolysis might either coagulate the sol or alter the electrical charge, causing a corresponding change in velocity of migration.

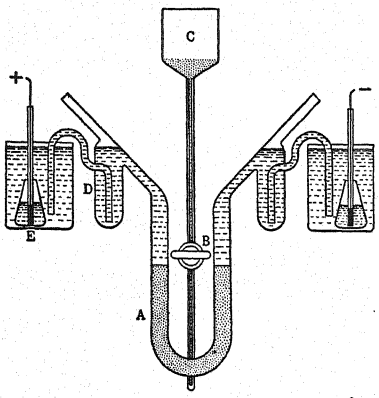


FIG. 51.—Svedberg's apparatus for conducting cataphoresis experiments.

(*b*) ELECTROENDOSMOSIS.—The theory of electroendosmosis has already been discussed in the early part of this chapter. *Electroendosmosis can be defined as the passage of liquid through a membrane or colloidal gel under the force of an applied electric current.* The direction of migration of the water through the membrane or gel is toward the pole which has the same sign as is possessed by the membrane or gel. Figure 52 shows diagrammatically in *A* a membrane inserted in a tube, together with the liquid levels in the arms of the tube, before the electric current has been applied. In Fig. 52 *B* is represented diagrammatically the relative position of liquid in the two arms of the tube after an electric current has been applied to the electrodes, assuming that the gel was negatively charged. The direction of flow accordingly indicates the sign of the charge on the interface. *The volume of flow is proportional to the electrokinetic potential existing across the double layer.*

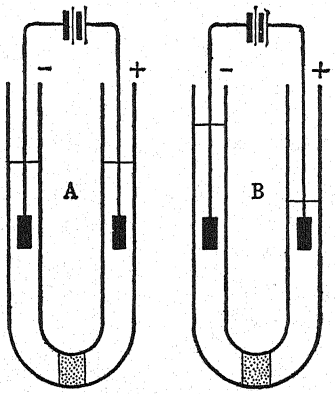


FIG. 52.—A diagrammatic representation of electroendosmosis.

The equations which apply in electroendosmotic measurements are for a single capillary tube,

$$v = \frac{r^2 \zeta H \epsilon}{4\eta l} \qquad (91)$$

where v = volume of flow;
l = the length of the capillary;
H = the e.m.f. applied across the diaphragm or between the electrodes, the other quantities being identical with those in equation (90),

and for a bundle of capillaries of cross section q,

$$v = \frac{q\zeta H\epsilon}{4\pi\eta l} \tag{92}$$

and

$$\zeta = \frac{4\pi v\eta l}{qH\epsilon} \tag{93}$$

If the liquid is not allowed to flow out but accumulates so as to produce a hydrostatic pressure P, the equations then become, for a single capillary

$$P = \frac{2\zeta H\epsilon}{\pi r^2} \tag{94}$$

and

$$\zeta = \frac{P\pi r^2}{2H\epsilon} \tag{95}$$

In these equations H, the applied e.m.f. has a different connotation from the E of the cataphoretic equation. H in reality is equal to El and is the fall in potential per centimeter multiplied by the length through which the fall takes place.

For a bundle of capillaries of cross section Q, we have

$$V = \frac{Q\zeta H\epsilon}{4\pi\eta l} \tag{96}$$

and

$$P = \frac{2\zeta El\epsilon}{\pi r^2} = \frac{2\zeta El\epsilon}{Q} \tag{97}$$

and

$$\zeta = \frac{\pi r^2 P}{2El\epsilon} = \frac{QP}{2El\epsilon} \tag{98}$$

The dimensions of the capillary (r and l or Q and l) can be eliminated from the endosmotic equations providing that the current i is kept constant. In such a case equations (86) and (87) apply.

Numerous papers have been published dealing with measurements involving electroendosmotic technic. Among the more interesting to the biochemist are those of Stamm,[28, 29] where sections of wood have been used as the membranes under investigation.

Most investigations on electroendosmosis have been conducted with water as the liquid medium. However, Strickler and Mathews[30]

[28] Stamm, A. J., Electroendosmose through Wood Membranes, Colloid Symposium Monograph, Vol. IV, pp. 246–257, Chemical Catalog Company, New York (1926).

[29] Stamm, A. J., Effect of Electrolytes on Electroendosmose through Wood Membranes, Colloid Symposium Monograph, Vol. V, pp. 361–368, Chemical Catalog Company, New York (1928).

[30] Strickler, A., and Mathews, H., Studies in Electric-Endosmose, *J. Am. Chem. Soc.*, 44: 1647–1662 (1922).

have investigated the behavior of various organic liquids, using membranes of filter paper.

Briggs [31] has compiled an excellent review of electroendosmosis, together with its application to industrial processes. Some of the industrial applications suggest interesting possibilities. Thus, it is pointed out that a considerable quantity of water can be removed from colloidal gels by passing an electric current through the gel. For instance, peat can be compressed into blocks, electrodes applied at the two ends of the block, and when a current is turned on, water will flow from the moist peat to the cathode. Only water mechanically held in a gel can be removed by such a process.

In a similar manner, clay can be collected and partially dewatered. If one has a thin suspension of clay, the clay particles can be made to collect on the anode, owing to cataphoretic migration, and later the mass of moist clay can be partly dewatered by subjecting the mass to conditions which will cause electroendosmotic flow. Briggs points out that Dawkins, in 1913, proposed a novel application of electroendosmosis to the process of brick manufacture. Wet clay has a marked tendency to adhere to a smooth metallic surface. In making wire-cut bricks, it is usual to employ a lubricant of some sort in order to prevent the clay from adhering to the cutting wires. It was found, however, that if the wires were connected with a source of direct current, the wire being made the cathode, the anode being inserted in the clay block, the clay would no longer stick to the wire, but that the wire would cut the clay cleanly, and with this electrical "lubrication" the power consumption was reduced by 25 to 30 per cent. The action is due to water collecting in a film on the surface of the cutting wires due to electroendosmotic flow, this water film then acting as a lubricant for the wire, the mass of clay itself never coming in contact with the metal.

Crowther and Haines [32] applied this method to a study of plowing. If the plowshare were made the cathode, being connected with an overhead power line, and the anode were imbedded in the soil of the field, it would be theoretically possible to lubricate the plowshare with a film of water so that the soil would never come in contact with the metal. They accordingly tested this theory, using both laboratory-scale experiments and actual plowing tests.

A slider, consisting of a weighted steel slab, was so arranged as to be drawn by weights across the surface of a plane of moist soil. The weight necessary to keep the iron block in steady motion was considered to be a measure of the friction of the block upon the soil surface. Figure 53 shows the results which were obtained. In the

[31] Briggs, T. R., Electrical Endosmose, Second Report on Colloid Chemistry, *Brit. Assoc. Advancement Sci.*, pp. 26–52 (1918).

[32] Crowther, E. M., and Haines, W. B., An Electrical Method for the Reduction of Draught in Ploughing, *J. Agr. Sci.*, 14: 221–231 (1924).

absence of electrification of the slab a weight of 0.6 kg. was necessary to keep the slab in motion. When the slab was made the anode, there was a sharp increase in the friction until at point *B* 1,500 grams were necessary to keep the slab in motion. At point *B* the current was shut off, the friction falling to point *C*. The higher friction at point *C* over that at point *A* was due to the drying of the surface, the moisture being drawn into the interior of the soil. At point *C* the iron slider was made the cathode. Moisture, now, was drawn from the soil to the surface of the moving iron plate, thus forming a lubricating film between the iron and the soil, the weight necessary to move the iron plate dropping rapidly to point *D*.

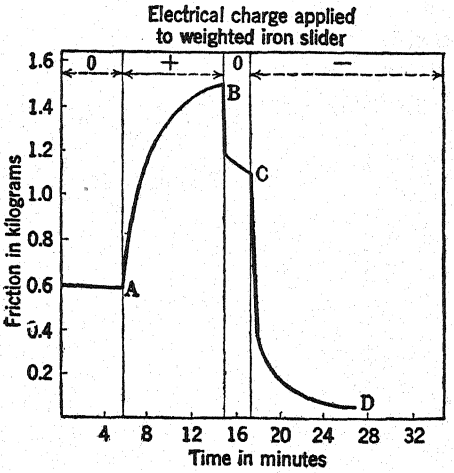


FIG. 53.—Showing how the friction between an iron slider and soil may be increased or decreased by electroendosmotic water flow.

Figure 54 shows a similar experiment in which the soil moisture content was varied and variations in electrical potential applied to the iron plate were introduced. It will be noted that the frictional force was reduced to approximately 20 per cent of its original value when soil containing 20 per cent moisture was used. When the soil contained only approximately 14 per cent of moisture, very little reduction in friction took place. Accordingly the moisture content of the material which is subjected to electroendosmosis becomes an important factor. It would appear that in the soil experimented with there was approximately 14 per cent of "bound water," *i.e.*, water adhering so firmly to the surface of the soil particles that it behaved as a part of the solid phase and could not undergo endosmotic flow.

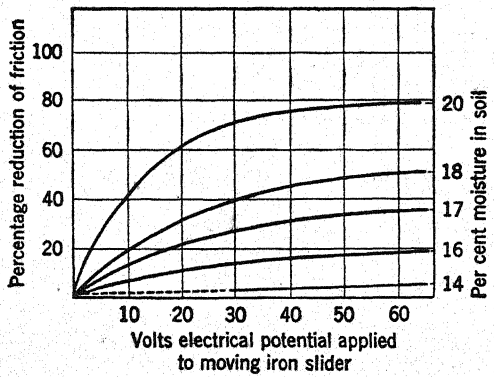


FIG. 54.—Showing the relationship between soil moisture and electroendosmotic water flow through the soil. (Data of Crowther and Haines.)

In the actual plowing experiments where the drawbar pull was recorded by means of a dynamometer, there was a reduction in the energy necessary for plowing, but the magnitude of the reduction was not sufficient to compensate for the current used.

(*c*) STREAMING POTENTIAL.—It is obvious that, if an electrical current induces a flow of liquid through a capillary, a condition which we have already discussed under electroendosmosis, the reverse phenomenon must take place, *i.e., forcing a liquid through a capillary will produce a difference in electrical potential between the two ends of the capillary.* The e.m.f. so induced is known as the *streaming potential.*

Kruyt,[33] Freundlich and Rona,[34] and Kruyt and Willigen[35] developed technics for the quantitative estimation of the streaming potential and equations for the calculation of the ζ-potential from the streaming potential. They used a single glass capillary and studied changes in the streaming potential produced by varying the kind of glass making up the walls of the capillary tube, as well as the effect of the various ions which are present in the liquid being streamed through the capillary. Kruyt found, using a glass capillary, that the electromotive force set up was directly proportional to the hydrostatic pressure and that the quantity H/P was a constant for a given capillary as long as the same solution was being forced through the capillary. Table XIX, taken from Kruyt's paper, illustrates this point.

Briggs[36] in an attempt to apply streaming potential methods to

TABLE XIX

THE E.M.F. SET UP BETWEEN THE TWO ENDS OF A GLASS CAPILLARY WHEN A MILLIMOLAR SOLUTION OF KCl IS FORCED THROUGH THE CAPILLARY UNDER VARIOUS PRESSURES

P, cm. mercury	H, millivolt	$\frac{H}{P}$
61.2	271	4.4
86.4	367	4.2
54.8	236	4.3
70.8	315	4.4

[33] Kruyt, H. R., Strömungspotentiale und Kolloidstabilität, *Kolloid Z.*, 22: 81–98 (1918).

[34] Freundlich, H., and Rona, P., Über die Beziehungen zwischen dem elektrokinetischen Potentialsprung und der elektrischen Phasengrenzkraft, *Sitz. preuss. Akad. Wiss.*, 20: 397–402 (1920).

[35] Kruyt, H. R., and van der Willigen, P. C., Strömungspotentiale und Kolloidstabilität, II. *Kolloid Z.*, 45: 307–319 (1928).

[36] Briggs, D. R., *J. Phys. Chem.*, 32: 641–675 (1928).

the determination of the electrokinetic charge on cellulose fibers observed that H/P was not a constant for different diaphragms of the same sample of cellulose. However, by introducing κ_s, the specific electrical conductivity of the liquid as it exists inside of the diaphragm and which is being streamed through the diaphragm, into the streaming potential equation in place of κ, the "bulk" specific conductivity of the liquid which was being streamed through the diaphragm, he [37] was able to obtain a constant ratio of potential difference to the pressure used to induce streaming. Briggs accordingly proposed the equation,

$$\zeta = \frac{4\pi\eta\kappa_s H}{P\epsilon} \tag{99}$$

where η = the viscosity of the liquid being streamed through the diaphragm;
P = hydrostatic pressure under which the liquid flows;
ϵ = the dielectric constant of the liquid;
κ_s = the specific electrical conductivity of the system, *i.e.*, the liquid as it exists in the pores of the diaphragm material;
H = the potential difference existing across the diaphragm.

The values that must be determined are P, H, and κ_s, and from these the electrokinetic potential can be calculated. The coefficient of viscosity for dilute aqueous solutions can be taken as 0.01. ϵ, the dielectric constant of water, is considered as having a value of 80. P, which is observed in centimeters of mercury, must be converted into dynes in order to express it in absolute units. Accordingly the height of the mercury column in centimeters must be multiplied by the specific gravity of mercury, 13.6, and the gravity constant, 981 dynes. H, which is read in millivolts, must be divided by 1,000 to reduce it to volts, and again divided by 299.86 to reduce volts to c.g.s. electrostatic units. κ_s observed in reciprocal ohms must be multiplied by 9×10^{11} to convert it into c.g.s. electrostatic units. The value of ζ so obtained would be in electrostatic units. In order to obtain this value in volts it must be multiplied by 299.86. Then

$$\zeta = \frac{H\kappa_s}{P} \times \frac{9 \times 10^{11} \times 4 \times 3.1416 \times .01 \times 299.86}{13.6 \times 981 \times 10^3 \times 299.86 \times 80}$$

$$= 1.0596 \times 10^2 \times \frac{H\kappa_s}{P}$$

where ζ is expressed in volts, H in millivolts, κ_s in reciprocal ohms, and P in centimeters of mercury.

[37] *Cf.* also Briggs, D. R., Surface Conductance, Colloid Symposium Monograph, Vol. 6, pp. 41–52, Chemical Catalog Company, New York (1928).

We have already noted that the term qd has been introduced into electrokinetics, and that the term qd is equivalent to the δe of equation (80). Substitute equation (80) in equation (99) and we have

$$\delta e = \frac{\eta \kappa_s H}{P} \tag{100}$$

All the terms on the right-hand side of equation (100) are measurable experimentally. The quantity δe, which may be regarded as *the electric moment per square centimeter of the double layer*, is an expression for the determination of the symmetry of the double layer in much the same way that the electric moment of a molecule is the expression of the symmetry of a molecule. Furthermore, this equation does not involve any assumptions which cannot be measured experimentally, and it is believed that it will be a much more valuable measure of the intensity of electrical forces at interfaces than is the ζ-potential as usually calculated.

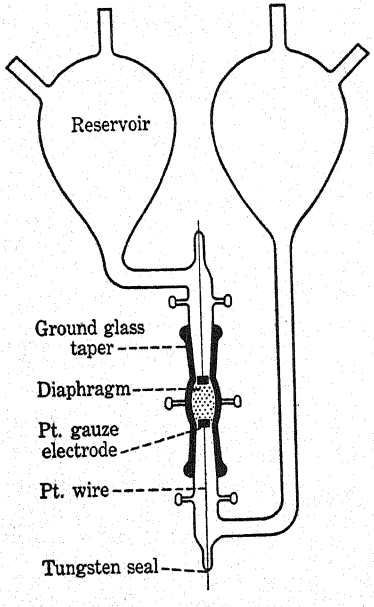


FIG. 55.—Solid-liquid streaming potential cell.

The apparatus devised by Briggs was later modified by Martin and Gortner [38] and still later by Lauffer.[39] Fig. 55 shows the latest model of the streaming potential cell. In this cell the electrodes are made of 80-mesh platinum gauze welded on to a platinum grid and are permanently mounted on the ends of the inside parts of the two standard taper-joints. Each electrode is held in place by the tension on the platinum wire which connects it to the tungsten seal at the end of the cell. By use of standard ground-glass joints mechanical clamps and gaskets have been eliminated. This cell can be inserted in the wiring diagram as described by either Briggs or Martin and Gortner. It is essential, however, that the potential which is set up by the liquid streaming between the

[38] Martin, W. McK., and Gortner, R. A., Studies on Electrokinetic Potentials. V. Interfacial Energy and the Molecular Structure of Organic Compounds. I. Electrokinetic Potentials at Cellulose-Organic Liquid Interfaces, *J. Phys. Chem.*, 34: 1509–1539 (1930).

[39] Lauffer, M. A., Electric Forces at Organic Liquid-Solid Interfaces, Ph.D. Thesis, University of Minnesota, June, 1937; *J. Phys. Chem.*, 42: (May, 1938).

two electrodes be measured by means of a quadrant electrometer. Use of a galvanometer causes polarization of the electrodes and may give rise to erroneous potentials.

Bull[40] has shown that gold or platinum electrodes used in this way in streaming potential cells give results identical with those obtainable when calomel half cells are used as the electrodes and that the gold or platinum ones have the advantage of avoiding a dangerous source of contamination with electrolytes.

Another type of streaming potential cell which is designed for the study of electrical forces at liquid-liquid interfaces was designed by Martin and used by Bull and Gortner.[41] This cell is shown in Fig. 56. The operation of the cell depends upon the streaming of a liquid through a small orifice in one electrode under sufficient pressure so that a "solid rod" of liquid (water or an aqueous solution) streams from that electrode and impinges upon the opposite electrode, the

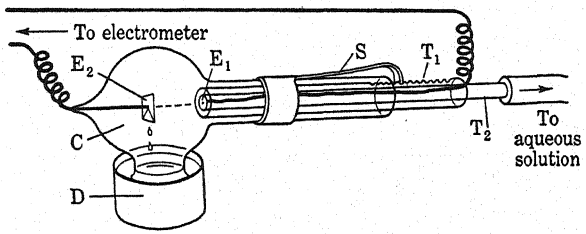


FIG. 56.—Oil-water streaming potential cell.

two electrodes being separated by the second immiscible liquid (oil) under investigation. Using this cell Bull and Gortner found that aqueous solutions of electrolytes streamed through paraffin oil yielded curves similar in shape to other curves resulting from similar studies of the effect of electrolyte solutions streamed through cellulose or quartz membranes, using the Briggs or Martin and Gortner type of apparatus. This liquid-liquid streaming potential apparatus should be of value in studying emulsification and the physical and chemical behaviors of different samples of oils where slight impurities, added components, or changes in structure make great differences in surface behavior.

(*d*) SEDIMENTATION POTENTIAL.—Dorn,[42,43] in 1878, discovered that when particles fall through a liquid, a difference of electrical

[40] Bull, H. B., Electrokinetics. XV. The Use of Inert Electrodes in Measuring the Streaming Potential, *J. Am. Chem. Soc.*, 57: 259–260 (1935).

[41] Bull, H. B., and Gortner, R. A., Electrokinetic Potentials. XI., *Physics*, 2: 21–32 (1932).

[42] Dorn, E., Über die galvanischen Ströme, welche beim Strömen von Flüssigkeiten durch Röhren erzeugt werden, *Ann. phys.*, 5: 20–44 (1878).

[43] Dorn, E., Über die Fortführung der Electricität durch Strömendes Wasser in Röhren und verwandte Erscheinungen, *Ann. phys.*, 9: 513–556; 10: 46–76 (1880).

potential is established between the top and the bottom of the liquid. This is the converse of cataphoresis in the same way that the streaming potential is the converse of electroendosmosis. In cataphoresis the particles are drawn through the liquid under the influence of an electric current. Accordingly *an e.m.f. is set up when particles are allowed to fall through a liquid.* Similar observations were made by Billitzer,[44] Freundlich and Mäkelt,[45] and Stock.[46,47] Stock allowed fine quartz powder to fall through nitrobenzene, ether, and toluene, and measured the magnitudes of the e.m.f. which was produced.

Bull [48] has applied this technic to the determination of the sign of the electrical charge on lead sulfide, and has attempted to explain the action of certain toxic ions in the industrial process known as ore flotation. Although he did not attempt to determine the absolute value of the electrokinetic potential on the galena particles, he was nevertheless able to show that galena possesses a positive charge when in contact with pure water and that certain ions may greatly increase whereas other ions cause a pronounced decrease in the magnitude of the potential.

In later studies Bull, Ellefson, and Taylor,[49] using cataphoretic technic, could not confirm the form of the curves which had been earlier secured by the sedimentation potential technic, and efforts in the author's laboratories to use the sedimentation potential as a quantitative method have so far resulted in failure to secure constant and reproducible data. Failure probably lies in the fact that it has so far been impossible to control exactly the path of the fall of the individual particles, so that the particles take a more or less random and irregular path in falling through the liquid, and the side motion of the particles, together with convection currents in the liquid, produce disturbing effects which interfere with a strict reproducibility of conditions.

For non-conducting liquids (or gases) the potentials developed through the Dorn effect may be of rather large magnitude. Stock measured potentials in excess of 80 volts for powders falling through organic liquids. Probably electrical effects in dust storms, where

[44] Billitzer, J., Über die Elektrizitätserregung durch die Bewegung fester Körper in Flüssigkeiten, *Ann. physik*, (4) 11: 937–956 (1903).

[45] Freundlich, H., and Mäkelt, E., Über den absoluten Nullpunkt des Potentials, *Z. für Elektrochem.*, 15: 161–163 (1909).

[46] Stock, J., Über die durch Bewegung fester Körper in Flüssigkeiten hervorgerufenen electroosmotischen Potentialdifferenzen, *Anzeiger Akad. Wiss. Krakau* [A.], (1913) 131–144.

[47] Stock, J., Beiträge zur Kenntnis der elektrischen Endosmose, *Anzeiger Akad. Wiss. Krakau* [A.], (1914) 95–106.

[48] Bull, H. B., The Electrostatics of Flotation, *J. Phys. Chem.*, 33: 656–658 (1929).

[49] Bull, H. B., Ellefson, B. S., and Taylor, N. W., Electrokinetic Potentials and Mineral Flotation, *J. Phys. Chem.*, 38: 401–406 (1934).

radio aerials often become highly charged and emit sparks, and instances where lightning bolts fall from a clear sky may be due to such origins.[50,51]

(*e*) THE ELECTROVISCOUS EFFECT.—Smoluchowski [52] noted in 1916 that the viscosity of a suspension of charged particles should be different from the viscosity of a similar suspension of uncharged particles due to the repulsion of charged particles possessing like signs, and similarly that the effect of electrolytes in altering the apparent viscosity might be accounted for to a considerable degree by the electrolytes altering the electrokinetic potential. He accordingly combined the electrokinetic equation with the Einstein equation (6) and derived the equation [53]

$$\frac{\eta}{\eta_0} = 1 + 2.5\phi\left[1 + \frac{1}{\kappa\eta_0 r^2}\left(\frac{\zeta\epsilon}{4\pi}\right)^2\right] \tag{101}$$

where κ = specific conductivity;
r = the radius of the particle;

and the other quantities have the connotations of the Einstein or electrokinetic equations. This equation expanded for the Kunitz equation (8) would be

$$\frac{\eta}{\eta_0} = \frac{1 + 0.5\phi}{(1 - \phi)^4} + \frac{1 + 0.5\phi}{(1 - \phi)^4}\left[\frac{1}{\kappa\eta_0 r^2}\left(\frac{\zeta\epsilon}{4\pi}\right)^2\right] \tag{102}$$

Bull [54] investigated the electroviscous effect for egg albumin and gelatin sols. He finds that the portion of equations (101) and (102) inside of the brackets may be ignored, since the values of this correction are only 2.15×10^{-3} for the egg albumin sols ($r = 2.18\text{m}\mu$) and 5.45×10^{-3} for gelatin sols ($r = 1.40\ \text{m}\mu$). As noted, he used the incorrect equation. The correct equation would make these values still lower.

(*f*) THE DETERMINATION OF THE CHARGE DENSITY ON THE SURFACE OF PARTICLES.—We have already noted that the ζ-potential involves not only the magnitude of the charge per unit area of the

[50] Gortner, R. A., Atmospheric Electricity During Sand Storms, *Science*, 70: 118–119 (1929).

[51] Shaw, P. E., Triboelectricity and Friction. IV. Electricity Due to Air-blown Particles, *Proc. Roy. Soc.* (*London*), (A), 122: 49–58 (1929).

[52] von Smoluchowski, M., Theoretische Bemerkungen über die Viskosität der Kolloide, *Kolloid Z.*, 18: 190–195 (1916).

[53] There is an unfortunate misprint in the final equation of Smoluchowski's paper where 2π is substituted for 4π. This misprint has been carried over into Freundlich's book (Kapillarchemie, Bd. II, p. 365, 1932) and into papers by du Noüy (du Noüy, L., *Ann. Inst. Pasteur*, 42: 742–769 1928) and Bull (Bull, H. B., *Z. physik. Chem.*, 161: 192–194, 1932).

[54] Bull, H. B., Bemerkungen zur Kryolyse lyophiler Kolloide, *Z. physik. Chem.*, 161: 192–194 (1932).

double layer but likewise the distance which separates the positive and negative charges in the double layer. That this is true has only recently been made clear, and accordingly nearly all colloid textbooks carry the statement that "electrolytes may reduce the charge to zero or may reverse it." What this statement really means is that electrolytes may reduce the ζ-potential to zero or may reverse it and that this may be brought about by an alteration in either charge or distance. As a matter of fact, the distance which separates the positive and negative charges in the double layer is usually the factor which is most affected. This was first shown in studies [55] of the behavior of electrolytes at a cellulose-water interface, using streaming potential technic. Figures 57, 58, and 59 illustrate the effect of KCl, $CaCl_2$, and $ThCl_4$ on the ζ-potential, the charge, and the thickness of the double layer at a water-cellulose interface. It is evident from these curves that the distance which separates the charges in the double layer is the factor which causes the decrease in the ζ-potential and that e (charge density per square centimeter of the double layer) is increasing with increasing salt concentration, while the double layer is collapsing. Apparently at a large value of e and a negligible value of δ the layer collapses and reverses. This is particularly shown in the curves for thorium chloride.

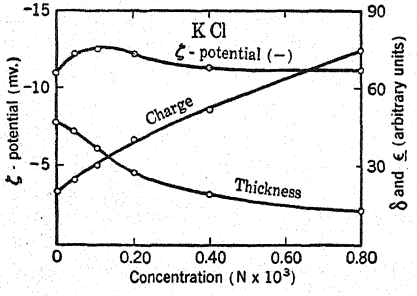


FIG. 57.—The effect of increasing concentrations of KCl on the ζ-potential, the thickness of the Helmholtz double layer, and the density of charge in the double layer at a cellulose-water interface. (Data of Bull and Gortner.)

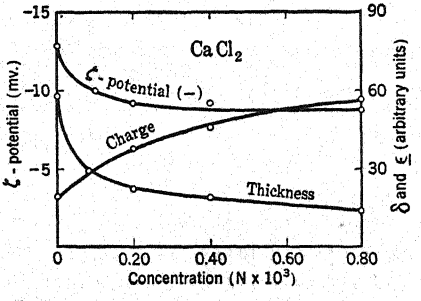


FIG. 58.—The effect of increasing concentrations of $CaCl_2$ on the ζ-potential, the thickness of the Helmholtz double layer, and the density of charge in the double layer at a cellulose-water interface. (Data of Bull and Gortner.)

It has been recognized for a long time that the flocculation of lyophobic sols by electrolytes may take place when there is still an appreciable ζ-potential. There is

[55] Bull, H. B., and Gortner, R. A., Studies on Electrokinetic Potentials. VI. Electrical Phenomena at Interfaces, *J. Phys. Chem.*, 35: 309–330 (1931).

accordingly a range about the isoelectric point within which lies the so-called critical zone [56] and a critical level of potential which has been called the critical threshold. This is shown in Fig. 60. The critical threshold is probably determined not so much by the charge at the interface as by the thickness of the double layer, and when this has been reduced to a given point, the system becomes unstable and flocculation begins. Here again the factor of *time* should be emphasized, since in a short time period flocculation may not be perceptible, whereas in a much longer time period flocculation may be complete. Thus the critical zone narrows with increasing time.

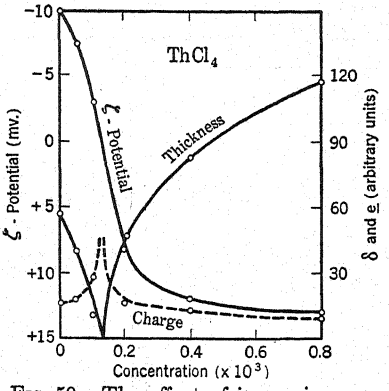


FIG. 59.—The effect of increasing concentrations of $ThCl_4$ on the ζ-potential, the thickness of the Helmholtz double layer, and the density of charge in the double layer at a cellulose-water interface. (Data of Bull and Gortner.)

The observation that charge density in the double layer increases with increasing electrolyte concentration is confirmed by Abramson [57] where he has calculated charge density on typhoid bacteria as a function of increasing salt concentration. Certain of his data are given in Table XX.

The form of the curves for NaCl, KCl, LiCl, etc., in electrokinetic studies at a cellulose-salt solution interface indicates that in all prob-

TABLE XX

CHANGES IN CHARGE DENSITY ON BACTERIA WITH INCREASING SALT CONCENTRATION
(Data of Abramson)

NaCl Concentration	ζ-Potential, Volts	Charge Density, e.s.u.
0.001 *M*	0.30	224
0.004	0.25	417
0.01	0.20	530
0.02	0.10	700
0.04	0.006	924

[56] Kruyt, H. R., Electric Charge and Limit Value in Colloids, *Proc. Acad. Sci. Amsterdam*, 17: 623–629 (1914); Sur le potentiel critique, *Rec. trav. chim.*, 39: 618–622 (1920).

[57] Abramson, H. A., The Relation of Electrical Charge of Bacteria to Their Stability, *J. Bact.*, 27: 89 (1934).

ability both anions and cations are being adsorbed into the double layer. For example, the lower concentrations of NaCl cause an increase in the ζ-potential from approximately -10 mv. to -14.10 mv. at a salt concentration of 1×10^{-4}. The ζ-potential then decreases to -10.90 mv. at a salt concentration of 1.6×10^{-3}. The increased negativity in all probability is due to an increased differ-

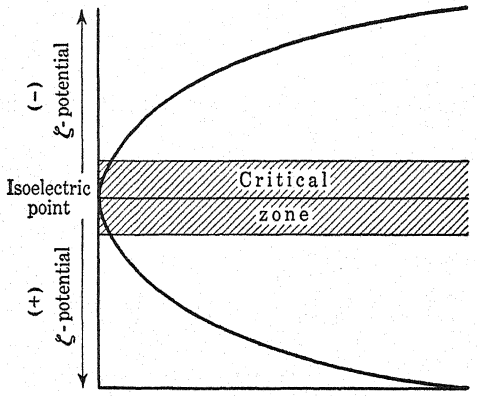


FIG. 60.—A diagrammatic representation of the relationship between colloid stability, the ζ-potential, and the "critical zone."

ential adsorption of anions into the double layer, whereas the decrease in the negativity which occurs later is in all probability due to more of the cations entering the double layer.

At the time Bull and Gortner made their studies no method was available for evaluating the absolute surface charge. More recently equations have been devised which make such evaluation possible. The generalized equation is [58]

$$\sigma = \sqrt{\frac{NDkT}{2000\pi}} \sqrt{\sum C_i \left(e^{-Z_i \frac{e\zeta}{kT}} - 1 \right) + \sum C_j \left(e^{+Z_j \frac{e\zeta}{kT}} - 1 \right)} \qquad (103)$$

where σ = the charge per unit area on the particle;

N = Avogadro's number;
D = the dielectric constant;
k = the Boltzmann constant;
T = the absolute temperature;
C = the concentration of electrolytes in moles per liter;
e = the elementary charge;
Z = the valence of the ions;

and i and j indicate the anion and cation, respectively.

[58] Abramson, H. A., Electrokinetic Phenomena, p 110.

When the valences of the ions making up the electrolyte are the same, equation (103) reduces to

$$\sigma = 2\sqrt{\frac{NDkT}{2000\pi}}\sqrt{C}\sinh Z\frac{e\zeta}{2kT} \qquad (104)$$

The importance of determining the charge density in electrokinetic studies is illustrated by the fact that an investigation [59] of the electrokinetic behavior of NaCl, KCl, $CaCl_2$, $MgCl_2$, and mixtures of NaCl : KCl, NaCl : $CaCl_2$, NaCl : $MgCl_2$, KCl : $CaCl_2$, KCl : $MgCl_2$, and $CaCl_2$: $MgCl_2$, at cellulose-aqueous solution interfaces, in an attempt to determine whether or not electrokinetic phenomena were concerned in the problem of ion antagonism, failed to demonstrate ion antagonism as reflected by the ζ-potential. Later a recalculation [60] of the data demonstrated the marked ion antagonism shown in Figure 61.

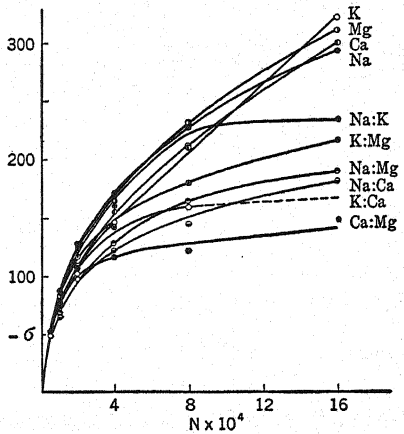


FIG. 61.—The relationships between charge density and salt concentration at a cellulose-water interface. Note ion antagonism exhibited by mixed salt solutions. (All salts as chlorides. Data of Bull and Gortner recalculated by Moyer and Bull.)

(*g*) THE COMPARISON OF VARIOUS ELECTROKINETIC TECHNICS.—It is perhaps pertinent at this point to inquire how electrokinetic measurements conducted by the various methods compare with each other. Briggs [61] in a study of the electrokinetic potential utilized the streaming potential method on egg albumin adsorbed on a quartz-water interface and compared his data with data which had been reported by Abramson [62] for the electrokinetic potential of egg albumin adsorbed on quartz determined by cataphoresis. The comparison was carried out through a *p*H range of 3.38–7.50. The two series of experiments lay essentially on the same curve, although less scattering was shown by the data obtained by the streaming potential method than by those

[59] Bull, H. B., and Gortner, R. A., Studies on Electrokinetic Potentials. VIII. Ion Antagonism, *J. Phys. Chem.*, 35: 700–721 (1931).

[60] Moyer, L. S., and Bull, H. B., Electrokinetics XVII. Surface Charge and Ion Antagonism, *J. Gen. Physiol.*, 19: 239–247 (1935).

[61] Briggs, D. R., The Measurement of the Electrokinetic Potential on Proteins by the Streaming Potential Method, *J. Am. Chem. Soc.*, 50: 2358–2363 (1928).

[62] Abramson, H. A., A New Method for the Study of Cataphoretic Protein Mobility, *J. Am. Chem. Soc.*, 50: 390–393 (1928).

obtained by cataphoresis. The fact that reproducible values were readily obtained in the streaming potential technic was proved by the results which were secured when three different diaphragms were studied at approximately the isoelectric point of the egg albumin. One diaphragm in contact with a dilute egg albumin solution of *p*H 4.78 gave a value of ζ of -0.55 mv. Another diaphragm at *p*H 4.66 gave a value of ζ of $+0.54$ mv. Still another diaphragm at *p*H 4.70 gave a value of ζ of 0.00 mv. This last diaphragm gave a value of ζ of $+4.56$ mv. at *p*H 4.57 and -2.63 mv. at *p*H 4.85. Apparently therefore the streaming potential and the cataphoretic equations were measuring identical electrical quantities.

Bull[63] compared the streaming potential technic, cataphoretic technic, and electroendosmotic technic on a Pyrex capillary and Pyrex particles coated with electrodialyzed gelatin or egg albumin. He found average ratios of 1.01 for ζ determined electroendosmotically and cataphoretically; 0.97 for ζ determined by the streaming potential technic and cataphoretically, and 0.99 for ζ determined by the streaming potential technic and electroendosmotically. These ratios indicate that at least for protein-covered surfaces and over the range of *p*H which was studied (*p*H 3.62–4.49) the three electrokinetic methods yield values which are within experimental error of one another.

He did not find a ratio of one to hold for bare quartz surfaces or for cellulose fibers and suggests that studies on surfaces which derive their charge by adsorption rather than by ionization are needed. A somewhat similar disagreement is noted by White, Monaghan, and Urban,[64] although they likewise report that when Pyrex particles are coated with gelatin the three methods yield comparable data. These authors report that the same values are given by cataphoretic and electroendosmotic technics with somewhat different values by the streaming potential technics where bare glass surfaces are studied, and suggest that in the cataphoretic and electroendosmotic technics we are dealing with an electrical pull, whereas in streaming potentials we have a pressure force which they believe does not deform the fixed double layer, that is, they suggest that only a diffuse portion of the double layer moves under hydrostatic forces, whereas both the diffuse and outer portions of the fixed double layer move in an electrical field.

It is possible that on these bare surfaces we have simultaneously an electrical migration of the particle and an electrodialysis induced by the imposed e.m.f., in which case, using cataphoretic and electro-

[63] Bull, H. B., Electrokinetics. XIV. A Critical Comparison of Electrophoresis, Streaming Potential, and Electrosmosis, *J. Phys. Chem.*, 39: 577–583 (1935).

[64] White, H. L., Monaghan, B., and Urban, F., The Influence of Electrolyte Concentration on the Ratio of Electrosmotic to Electrophoretic Mobilities, *J. Phys. Chem.*, 39: 611–622 (1935); A Comparison of the Electrophoretic, Electrosmotic and Stream Potential Isoelectric Points at Glass and Gelatin Surfaces, *J. Phys. Chem.*, 39: 585–591 (1935).

endosmotic technics, the absolute density of charge on the surface might be progressively changing, whereas with streaming potential technic the current density developed may not be sufficiently high to induce electrodialysis. Additional studies which would serve to clear up the behavior of bare surfaces are greatly needed.

(*h*) CAPILLARY SIZE AND ELECTROKINETIC BEHAVIOR.—Komagata [65] considered the influence of capillary size on the streaming potential and concluded that there was a limiting radius of the capillary below which the true streaming potential would not be observed. This is due in part to the change in the thickness of the double layer with change in ionic concentration and in part to endosmotic effects which result from the streaming potential. Thus, if a given pressure is applied across a diaphragm, a streaming potential is developed, and this developed streaming potential may induce an endosmotic flow countercurrent to the original direction of streaming, so that the effective pressure is less than the hydrostatic pressure which is applied.

Bull and Moyer [66] have considered Komagata's equations and the electrical back pressure and concluded that the radii of the capillaries in the cellulose and quartz membranes studied by Bull and Gortner lie outside of the critical radius. Bull [67] developed equation (105) for describing electrical back pressure.

$$\frac{P}{P_e} = \frac{\eta \kappa_s r^2}{8(\mu)^2} + 1 \qquad (105)$$

where P = the applied hydrostatic pressure;
P_e = the electroendosmotic back pressure;
r = the radius of the capillary;
η = the viscosity of the liquid;
μ = the electric moment (δe) of the double layer;
κ_s = the specific conductivity of the liquid as it exists in the capillary.

He points out that the difficulties which some workers have experienced are unquestionably due to their working with capillaries which were below the critical radius. In electrolyte solutions the double layer is reduced in thickness, and accordingly valid results may be obtained at electrolyte concentrations of the order of 10^{-4}. When working with more dilute aqueous solutions or with organic liquids, particularly if the systems possess a high electrokinetic potential or are

[65] Komagata, S., On the Equations of Streaming Potential and Electroendosmosis, *Researches of the Electrotechnical Laboratory*, No. 362, Tokyo, Japan, 49 pp., (1934).

[66] Bull, H. B., and Moyer, L. S., Electrokinetics. XVI. Streaming Potential in Small Capillaries, *J. Phys. Chem.*, 40: 9–20 (1936).

[67] Bull, H. B., Die Bedeutung der Kapillarenweite für das Strömungspotential, *Kolloid Z.*, 60: 130–132 (1932).

characterized by low specific conductances, special precautions must be taken to see that the results which are obtained are not affected by too small capillary radii. The cellulose diaphragms which were above the critical radius for dilute solutions of electrolytes have been found to be below the critical radius for certain pure organic liquids.

(*i*) APPLICATIONS OF ELECTROKINETIC TECHNICS.—Abramson's book lists many applications in the fields of biology and medicine. Only a few additional examples will be noted here.

Moyer has studied extensively the electrophoretic mobility and isoelectric points of cholesterol sols which were prepared by various methods. He found no concordance in the literature as to the electrokinetic behavior of such sols, although they had been investigated by various workers.[68-73] Keeser reported an isoelectric point $< p$H 1.3, Eagle an isoelectric point of pH 2.1–3.6, and Remesow[74] an isoelectric point of pH 3.2. Remesow's description of the preparation of his sols was not sufficiently explicit to permit duplication of his technic. However, Moyer[75] attempted to repeat the experiments reported by other workers and secured the data shown in Table XXI. He then devised a new technic and prepared cholesterol sols by grinding crystals of cholesterol with ice in an agate mortar in a room maintained at $-10°$ C. All sols so prepared showed an isoelectric point of pH 3.0–3.3 with a velocity at pH 5.8 of 1.47–1.55 μ/sec. When such sols were boiled for two hours, the isoelectric point dropped to approximately pH 1.7 and the velocity increased to approximately 2.44 μ/sec.

The above experiments strikingly illustrate the divergent results which can be obtained when different methods are used to prepare colloidal sols. In all the methods noted in Table XXI where solvents

[68] Keeser, E., Studien über Cholesterin und seine Ester, *Biochem. Z.*, 154: 321–327 (1924).

[69] Porges, O., and Neubauer, E., Physikalisch-chemische Untersuchungen über das Lecithin und Cholesterin, *Biochem. Z.*, 7: 152–177 (1907).

[70] Stern, R., Über die klinische Bedeutung des Cholesterins in der Galle und im Blutserum, *Arch. Exptl. Path. Pharmakol.*, 112: 129–175 (1926).

[71] Rona, P., and Deutsch, W., Untersuchungen über Cholesterin- and Lecithinsuspensionen, *Biochem. Z.*, 171: 89–118 (1926).

[72] Kermack, W. O., and MacCallum, P., The Influence of Gelatin on the Stability of a Colloidal Solution of Cholesterol, and on the Charge on the Particles, *Proc. Roy. Soc.* (*Edinburgh*), 45: 71–89 (1925).

[73] Eagle, H., Studies in the Serology of Syphilis. III. Explanation of the Fortifying Effect of Cholesterin upon the Antigen as Used in the Wassermann and Flocculation Tests, *J. Exptl. Med.*, 52: 747–768 (1930).

[74] Remesow, I., Physikalische-chemische Untersuchungen über den kolloidalen Zustand des Cholesterins, Cholesterinesters und Lecithins, I-IV, *Biochem. Z.*, 218: 86–172 (1930).

[75] Moyer, L. S., Untersuchungen über die Elektrophorese der Cholesterinsuspensionen, *Biochem. Z.*, 273: 122–131 (1934); Electrophoresis of Sterols, II. Ergosterol, *J. Gen. Physiol.*, 18: 749–753 (1935); Electrophoresis of Sterols. III. Further Investigations of Cholesterol Surfaces, *J. Gen. Physiol.*, 19: 87–94 (1935).

TABLE XXI

PROPERTIES OF CHOLESTEROL SOLS PREPARED BY DIFFERENT METHODS FROM SAME CHOLESTEROL

Method	Solvent	Heated, hours	Temp., ° C.	Isoelectric Point, *p*H	Velocity at *p*H 5.8, μ/sec.	Remarks
Keeser	Alcohol	0.75	100	1.3	2.35	
"	Alcohol	0.25	100	1.9	2.83	
"	Alcohol	2.00	100	1.3	2.56	
"	Acetone	2.00	100	2.4	1.84	
Porges-Neubauer	Acetone	7.0	50–60	1.7	2.37	
" "	Acetone	24.0	40	...	2.33	
Stern	Alcohol	24.0	85	2.4	3.27	
"	Alcohol	5.5	85	...	2.85	
"	Alcohol	1.0	85	...	3.61	
"	Benzine	1.0	85	...	2.89	Air
Rona-Deutsch	Acetone	2.0	60–70	1.2–1.3	3.12	bubbled
" "	Acetone	1.0	60–70	...	4.00	through
" "	Acetone	0.75	60–70	...	4.50	
Kermack-MacCallum	Alcohol	0.05	65	2.8	2.35	0.2% alcohol
" "	Alcohol	0.05	65	2.1	1.97	5.0% "
Eagle	Alcohol	0.00	...	3.00	2.30	
"	Dioxan	0.00	...	2.70	2.50	

were employed there appears to be a portion of the solvent remaining adherent to the surface of the cholesterol particles, thus affecting the electrokinetic behavior of the material.

Moyer [76] studied the species relationships in *Euphorbia*, as shown by the electrophoresis of latex particles derived from the plant sap, and shows that the various taxonomic groups in twenty-one species of *Euphorbia* can be differentiated by the form of the electrophoretic curves of the latex particles. In some species the isoelectric point of the latex particles lay close to *p*H 3.0. In others it was near *p*H 4.7. Apparently in the former the latex possessed a sterol surface and in the latter a protein surface. In one taxonomic group (the poinsettias) he found a marked difference in the form of the curves and in the isoelectric point for the latex particles of *E. heterophylla*. Figure 62, taken from Moyer's data, shows this divergent curve. On investigation of the nature of the nucleus, *E. heterophylla* was found to be a tetraploid form possessing 56 chromosomes, whereas all the others in this botanical group possessed the diploid number of 28 chromosomes.

[76] Moyer, L. S., Species Relationships in *Euphorbia* as Shown by the Electrophoresis of Latex, *Am. J. Botany*, 21: 293-313 (1934); Electrophoresis of Latex and Chromosome Numbers of Poinsettias, *Botan. Gaz.*, 95: 678–685 (1934).

Later Moyer [77] studied the constancy of the latex isoelectric points and compared data on latex from plants grown from seed in Minnesota in 1933–1934 with similar data from plants grown from seed in Pennsylvania during 1932–1933, and in all instances found that the maximum deviation of the isoelectric point did not exceed 0.1 *p*H. In a study of the electrophoretic behavior of the latex of *Asclepias*, he [78] could not distinguish between those forms having 22 or 24 chromosomes. However, he did find constant species differences in the isoelectric points and also constant species differences in the form of the electrophoretic curves. In all cases the surface of the latex in *Asclepias* appeared to be protein coated. He accordingly suggests that electrokinetic studies may provide a useful tool for the taxonomist.

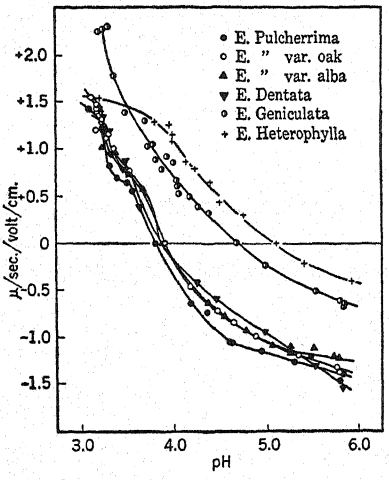


FIG. 62.—Electrokinetic curves *vs.* *p*H for the latex particles of various species of *Euphorbia*. (Data of Moyer.)

Sen [79] inserted microelectrodes into the root hairs of *Azolla pinnata* and the petiole hairs of *Urtica dioica* and found that the protoplasmic granules were negatively charged and that the velocity under which they migrated was independent of their size and shape.

In a study of bacteria at various stages in the growth cycle,[80] young cells of *Escherichia coli* up to seven hours of age had a higher mobility than had older cells, and the "rough" and "smooth" cultures likewise differed in mobility.

Martin [81] used the streaming potential technic to study the identity or non-identity of various protein fractions extracted from wheat flours

[77] Moyer, L. S., The Constancy of Latex Isoelectric Points, *Protoplasma*, 21: 588–593 (1934).

[78] Moyer, L. S., Chromosome Numbers and Electrophoresis of Latex in Asclepias, *Botan. Gaz.*, 97: 860–865 (1936).

[79] Sen, B., The Electric Charge of the Colloid Particles of Protoplasm, *Ann. Botany*, 48: 143–151 (1934).

[80] Moyer, L. S., Changes in the Electrokinetic Potential of Bacteria at Various Phases of the Culture Cycle, *J. Bact.*, 32: 433–464 (1936).

[81] Martin, W. McK., Electrokinetic Properties of Proteins. I. Isoelectric Point and Solubility of Wheat Proteins in Aqueous Solutions of Ethanol, *J. Phys. Chem.*, 35: 2065–2090 (1931); II. Adsorption of Gliadin at Glass-Liquid Interface as Influenced by Hydrogen-ion Concentration, *J. Phys. Chem.*, 38: 213–229 (1934).

and found the method to yield information which could not be obtained by the usual technics of the cereal chemist.

An application in the field of industry is the observation [82] that latex particles could be "electroplated" by cataphoresis upon metal, cloth, or wooden surfaces. This observation of the electrodeposition of rubber has created a large industry.

[82] Sheppard, S. E., The Electrical Deposition of Rubber, *Trans. Am. Electrochem. Soc.*, 52: 47–82 (1927).

CHAPTER VII

SURFACE TENSION, INTERFACIAL TENSION, SURFACE ENERGY, AND ADSORPTION

We have already discussed under emulsions certain phenomena which are dependent upon surface tension or interfacial tension. We have noted that an efficient emulsifying agent is one which lowers the interfacial tension between the two mutually insoluble phases. In order, however, to discuss adequately certain fundamental properties of colloid systems, it is necessary to consider in somewhat more detail the forces which operate to produce the phenomena which are designated as surface tension and interfacial tension. Such a discussion will necessarily be limited to the barest outline which will be adequate to lay the foundation for the discussions which are to follow.

Willows and Hatschek [1] have presented a most excellent discussion of surface tension and surface energy and their influence on chemical phenomena. The reader is referred to their book for a more complete elaboration of the theory. Likewise, Rideal [2] and Adam [3] have devoted a considerable part of their books to these questions.

SURFACE TENSION.—The energy of a gas is due solely to the kinetic energy of the gas molecules. Owing to kinetic energy, the gas molecules are in constant motion, and an incessant bombardment of an individual gas molecule by the surrounding molecules takes place. As the gas molecules come closer together, owing to compression, the chances of collision are increased per unit of mass. Consequently the energy of a gas is increased as the volume decreases and is diminished as the volume increases, the mass being kept constant. Accordingly as we allow a gas to expand so as to increase its volume, the net energy of the system is decreased. Conversely, compressing a gas so as to decrease its volume causes an increase in the energy of the system.

[1] Willows, R. S., and Hatschek, E., Surface Tension and Surface Energy, Third Edition, J. and A. Churchill, London (1923).

[2] Rideal, E. K., An Introduction to Surface Chemistry, Second Edition, University Press, Cambridge, England (1930).

[3] Adam, N. K., The Physics and Chemistry of Surfaces, Oxford University Press (1930).

A liquid differs from a gas in that its molecules are closer together and it is capable of assuming a definite form, *i.e.*, it has a boundary surface. A gas always completely fills the container in which it is placed, no matter how large the container may be. It is in this respect that it differs from a liquid which occupies a more or less fixed volume. A liquid possesses kinetic energy similar to a gas, but since it has a surface, it likewise possesses surface energy, and it is this surface energy which confers upon liquids many of the properties which are not possessed by gases.

The surface of a liquid differs from the body of the liquid, in that the molecules making up the surface are largely oriented in some particular direction. The molecules in the body of the liquid are largely distributed at random, but the work of Hardy, Harkins, Langmuir, Adam, and others has shown that the molecules in the surface film of liquids are in general arranged in an orderly fashion. This question of molecule orientation will shortly be discussed at length. Suffice it to say here that the surface of a liquid behaves as if there were a "skin" drawn over the bulk of the liquid, differing in physical properties and in molecular arrangement from the bulk of the liquid beneath the surface layer. The concentration of molecules per unit area in this surface layer is usually greater than in an equivalent volume within the bulk of the liquid. The layer of molecules on the surface is more or less rigid and gives rise to the phenomenon which we call surface tension.

Surface tension is due to molecular cohesion. According to LaPlace, molecules in a liquid have a pronounced attraction for one another. This attractive force, however, operates over only a short distance and is greatest at not to exceed one or two molecular diameters. It is probably negligible at a distance of 5mμ but is quite large as we approach one molecular diameter. Figure 63 shows diagrammatically the force which must be overcome in bringing a molecule from the interior of the liquid phase into the surface of the liquid and from the surface of the liquid into the vapor phase. We have at *A* within the body of the liquid a molecule which is attracted equally from all sides by other molecules. However, as this molecule approaches and touches the surface at *B*, it is held back from escaping into the vapor

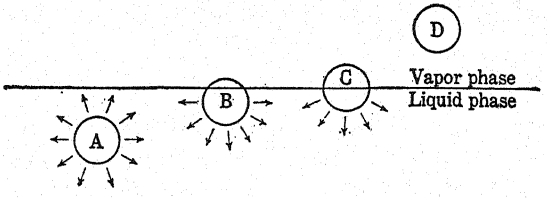


FIG. 63.—A diagrammatic representation of the direction and degree of the forces of molecular attraction acting upon a molecule, *A* in the body of the liquid, *B* touching the surface, *C* in the interface, and *D* in the vapor phase.

phase by the molecular cohesion with the surrounding molecules. The arrows in the diagram show the direction of this cohesional attraction. At *A* there is an equal pull in all directions; at *B* there is an excess of downward and lateral pull. In order for the molecule to reach the interfacial film at *C*, energy must be expended to overcome a part of the downward and lateral attractions, and a further amount of energy must be expended to overcome the downward pull when the molecule passes from *C* into the vapor phase at *D*. This downward and lateral pull of molecules which are in the surface film or which are entering the surface film is the tension which we measure and call surface tension. An excellent example to show surface tension is diagrammatically represented in Fig. 64. A wire loop, *ABCD*, is constructed and a movable crossbar, *EF*, of negligible weight, is placed upon this loop. If the area, *BCEF*, is then covered with a liquid film, the contractile force of the film will lift crossbar *EF* until it approximates contact with the wire *BC*. If now additional weights are added at *G*, a weight will be found which will just stretch the film *BCEF* indefinitely (until the film breaks). If the weight (gm./980.1) added and the length (cm.) of the bar, *EF*, in contact with the film are known, and we divide half the weight (2 surfaces) by this length, we obtain *surface tension per unit length*. The surface tension per unit of length is independent of the surface area, and this fact differentiates the surface tension from the tension of an elastic film. (In an elastic film it is obvious that a given weight will stretch the film to a new equilibrium position and that an additional weight will stretch it to a second equilibrium position, etc.)

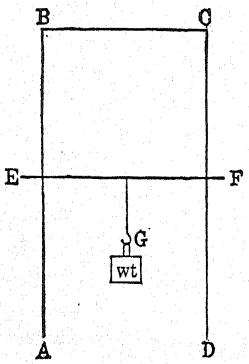


FIG. 64.—A diagrammatic representation of the fact that work must be done to increase surface area.

In the film in question we are dealing with two surfaces, one for each side of the film. The work done, expressed in ergs, to increase a surface by 1 sq. cm. is numerically equal to the surface tension. Surface tension is usually expressed in ergs per square centimeter, where an erg is the work done when 1 dyne moves the point to which it is applied through a distance of 1 cm. parallel to the direction of the force.

Table XXII shows the surface tension of certain common liquids. *Surface tension decreases with an increase in temperature and becomes zero at the critical temperature.* The decrease in surface tension is directly proportional to the temperature except possibly for a short range close to the critical temperature.

Referring again to Fig. 63, we have noted that energy is expended

TABLE XXII

SURFACE TENSION (γ) IN c.g.s. UNITS FOR CERTAIN LIQUID-AIR INTERFACES

	Temperature, ° C.	γ		Temperature, ° C.	γ
Chloroform	25	26.2	Glycerine	20	63.4
Methyl alcohol	20	22.61	Mercury	0	480.3
Ethyl alcohol	20	22.27	Hydrogen peroxide (56.06%)	0	77.46
Ethyl ether	20	17.10	Water	0	75.64 ± 0.1
Acetic acid	20	27.63		10	74.22 ± 0.05
Benzene	20	28.86		20	72.75 ± 0.05
Benzaldehyde	20	40.04		25	71.97 ± 0.05
Aniline	20	42.58		30	71.18 ± 0.05

in bringing molecules into the surface film and in removing a molecule from the surface film into the vapor phase.

Stefan,[4] in 1886, proposed a generalization known as Stefan's law, in which he stated that one half of the latent heat of vaporization of a liquid was expended in pulling a molecule of the liquid into the surface film, the remainder being expended in pulling it out of the surface film into the vapor phase.

For example, the following processes are necessary to vaporize a molecule of water:

1. The water molecule must be brought from the body of the liquid to the surface layer.

2. The molecule must be pushed into and become a part of the surface layer of molecules.

3. In order to vaporize this molecule, it must be forced out of the surface layer of molecules into the vapor phase.

The latent heat of vaporization of water is 540 calories at 100° C. Five hundred calories are expended in overcoming the pull of the neighboring molecules of liquid water. Only 40 calories are used to overcome the vapor density, atmospheric pressure, etc., *i.e.*, 40 calories represent the work done against the vapor phase, the remainder of the heat being utilized to overcome the downward pull of the liquid phase. If Stefan's law were strictly correct, we would have for water one-half of the quantity, 21×10^9 dynes per gram of water, as the force necessary to bring the molecules of 1 gram of water into the surface film. Since 10^6 dynes is equivalent to 1 atmosphere pressure, we would have

[4] Stefan, J., Über die Beziehung zwischen den Theorien der Capillarität und der Verdampfung, *Wied. Ann.*, 29: 655–665 (1886).

$\frac{0.5(21 \times 10^9)}{10^6}$, or 10,500 atmospheres pressure as the cohesional pressure of 1 gram of water.

Harkins and Roberts [5] pointed out that *Stefan's law is only a rough approximation,* and that associated liquids and non-associated liquids differ markedly in their surface energy relations. Table XXIII shows certain of their data. It will be noted that, instead of half of the energy of vaporization being expended in pulling the molecule out of the surface film into the vapor phase as required by Stefan's general-

TABLE XXIII

SHOWING FOR SOME COMMON LIQUIDS THE INTERNAL LATENT HEAT OF VAPORIZATION PER MOLECULE (λ) AND THE WORK EXPENDED (e) IN BRINGING A MOLECULE FROM THE INTERIOR OF A LIQUID INTO THE SURFACE FILM AND (j) IN CAUSING THE MOLECULE TO "JUMP" FROM THE SURFACE FILM INTO THE VAPOR PHASE. THE ENERGY VALUES ARE EXPRESSED IN 10^{-14} ERG (DATA OF HARKINS AND ROBERTS)

Liquid	Absolute Temperature, Degrees	(λ)	(e)	(j)	$\left(\frac{e}{\lambda}\right)$ Per Cent	$\left(\frac{j}{\lambda}\right)$ Per Cent
Carbon tetrachloride.	298	50.72	17.1	33.6	33.70	66.30
Ethyl ether.....	283.9	41.71	12.7	29.0	30.46	69.54
Benzene.....	298.1	52.8	19.5	33.3	36.93	63.07
Chlorbenzene.....	298	65.1	21.9	43.2	33.63	66.37
Ethyl acetate.....	303	53.1	16.0	37.1	30.13	69.87
Nitrogen.....	70	8.67	3.84	4.83	44.29	55.71
Oxygen.....	70	10.81	4.50	6.31	41.63	58.37
Methyl alcohol.....	413	43.2	10.1	33.1	23.38	76.62
Ethyl alcohol.....	383	55.6	11.7	43.8	21.04	78.77
Acetic acid.....	423	34.1	11.8	22.3	34.60	65.40
Water.....	283	69.6	10.5	59.1	15.09	84.91
Mercury.....	313	96.3	46.6	49.7	48.40	51.60

ization, the per cent energy expenditure for this process ranges from 55.71 for liquid nitrogen to 84.91 for water. Accordingly the calculated energy necessary to bring 1 gram of water into the surface film, as noted above, is in error.

Harkins and Roberts give the total energy necessary for the vaporization of a molecule of water at 10° C. as 69.6×10^{-14} erg. Of this amount, 10.5×10^{-14} erg was expended in drawing the molecule from the interior of the liquid into the surface film, and 59.1×10^{-14} erg was expended in moving the molecule from the surface film into the

[5] Harkins, W. D., and Roberts, L. E., Vaporization in Steps as Related to Surface Formation, *J. Am. Chem. Soc.*, 44: 653-670 (1922).

vapor phase. These values are widely divergent from the 50 : 50 ratio suggested by the generalization of Stefan.

Accordingly we can calculate the maximum surface area which can be occupied by 1 gram of water molecules at 10° C. Taking 0.37674×10^{10} ergs as representing the energy necessary to raise 1 gram of water from 10° C. to 100° C., and since 2.26×10^{10} ergs is the energy required to vaporize 1 gram of water at 100° C., we have 2.636×10^{10} ergs as the energy necessary to evaporate 1 gram of water at 10° C.

Of this amount, 15.09 per cent is expended in bringing the molecules from the interior of the liquid into the surface film. Accordingly, taking 71.94 ergs as the energy necessary to form 1 sq. cm. of water surface at 10° C., we have 15.09 per cent of $\frac{2.636 \times 10^{10}}{71.94}$, or 55,292,000 sq. cm., as the area of surface film which can be covered at 10° C. by 1 gram of water or a film 0.18 mμ thick.

The energy to remove a molecule from a surface film into the vapor phase is to some extent dependent upon the form of the surface. In Fig. 65 we have 3 molecules, molecule *D* in a concave surface, molecule *E* in a plane surface, and molecule *F* in a convex surface, the center of the molecule being spaced at an equal distance from the surface. More of the molecule protrudes from the surface at *F* than at *E*, and more at *E* than at *D*. Accordingly there is the least cohesional downward and lateral pull of adjacent molecules on the molecule at *F*, and the greatest downward and lateral pull on the molecule at *D*. The molecule at *F* can escape from the surface with less expenditure of energy than molecule *E*, and molecule *E* can escape with less energy expenditure than molecule *D*. Accordingly molecules will spontaneously evaporate from surface *F* to surface *E*, and from surface *E* to surface *D*. This difference in surface energy relationships becomes important when we are dealing with very minute particles or droplets. The small droplets with a highly curved surface, as in *F*, will spontaneously evaporate to larger droplets having more nearly plane surfaces. Correspondingly, a small particle having a highly curved surface will have a greater solubility in a given liquid than will a large crystal of the same material. As we shall see later, this is an important consideration when we are dealing with particles of colloidal dimensions.

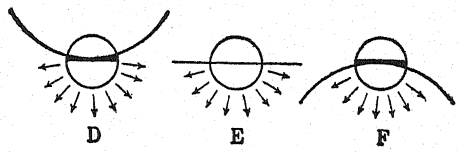


FIG. 65.—A diagrammatic representation of the direction and the intensity of the forces of adhesion acting upon a molecule in concave, convex, and plane surfaces.

INTERFACIAL TENSION.—Interfacial tension differs from surface tension only in that surface tensions refers to a liquid-vapor interface,

whereas interfacial tension refers to liquid-liquid or solid-liquid interfaces. No satisfactory method has yet been devised for measuring accurately interfacial tensions existing at solid-liquid interfaces. The interfacial tension of liquid-liquid interfaces can, however, be very conveniently measured by technic similar to that which is used for measuring the tension existing at liquid-vapor boundaries.

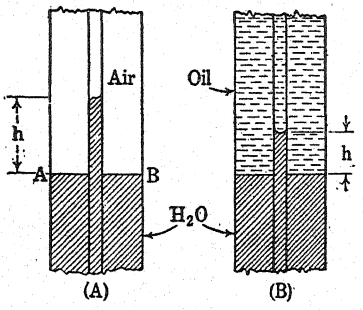


FIG. 66.—A diagrammatic representation of the relationship between surface tension and interfacial tension as measured by the capillary-tube method.

One of the earliest methods for the measurement of surface tension involved the determination of the height to which a liquid would rise in a capillary tube inserted into a plane surface in the liquid. Diagram *A*, Fig. 66, shows such a measurement. If we have a plane surface *AB* of a liquid in an open vessel, and into this plane surface a capillary tube is inserted and the liquid wets the wall of the capillary tube, the liquid will rise in the capillary, owing to a difference in curvature of the surface within the capillary, since the vapor pressure at equilibrium with a concave liquid surface within the capillary is smaller than the vapor pressure on the plane surface. Accordingly the liquid rises in the capillary to a height, h, which is equal to the difference in vapor pressure plus the weight of a column of liquid of height h, which will compensate for the difference in vapor pressure of the liquid within the capillary. The surface tension of the liquid can accordingly be calculated from the equation:

$$\gamma' = \tfrac{1}{2} hrD \qquad (106)$$

where γ' = surface tension in grams per square centimeter;
h = height, in centimeters, of the rise of the liquid in the capillary;
r = the radius of the capillary tube, in centimeters;
D = the specific gravity of the liquid in grams per cubic centimeter.

In order to convert γ' into γ, the surface tension, *i.e.*, into dynes or ergs per square centimeter, it is necessary to multiply by 980.1, the gravity constant.

Diagram *B*, Fig. 66, indicates how a similar arrangement can be used to measure interfacial tension (*cf.* Pound[6]). The interfacial ten-

[6] Pound, J. R., Interfacial Tension, *J. Chem. Soc.*, 123: 578–599 (1923).

sion at the interface of two immiscible liquids can be calculated by the equation

$$\gamma = \frac{1}{2} Gr\lambda\mu \left(h + \frac{r}{3\mu}\right)(D - D') \qquad (107)$$

where γ = interfacial tension in ergs per square centimeter;
G = 980.1 dynes;
r = radius of the capillary tube in centimeters;
λ = the ratio of the difference in level in the two liquids when the outer tube is of large diameter and the outer tube is of small diameter;
μ = the value of the scale division in centimeters;
h = difference in level of the two interfaces (the highest to the lowest points of the meniscus) inside and outside the capillary tube, in scale divisions;
D and D' = density of the two liquids, respectively.

Table XXIV shows certain values of the interfacial tension of pure liquids against a water surface, taken from the data of Pound. In a later paper, Pound[7] determined the interfacial tension between 42 organic liquids and water or various aqueous solutions. He points out that the greater the mutual solubility between the two liquids, the

TABLE XXIV

INTERFACIAL TENSION BETWEEN CERTAIN PURE LIQUIDS AND WATER
(Data of Pound)

	Interfacial Tension, 30° C.	Interfacial Tension, 10° C.
Benzene	32.5	34.10
Ether	11.13	10.19
Chloroform	31.41	32.17
Carbon tetrachloride	42.75	.....
Carbon disulfide	46.31	.....
Toluene	34.6	.....
Aniline	6.00	.....
Nitrobenzene	24.1	.....
Turpentine	23.0	.....
Para aldehyde	9.6	.....
Amyl alcohol	4.89	.....
Amyl acetate	10.88	.....
Ethyl acetate	6.27	.....
Cresol	4.28	.....

[7] Pound, J. R., Interfacial Tensions between Organic Liquids and Water or Aqueous Solutions, *J. Phys. Chem.*, 30: 791-817 (1926).

lower the interfacial tension between them. He also notes that the interfacial tension between certain esters in contact with dilute acid or alkali solutions in water decreases with time, and ascribes this decrease to the hydrolysis of the esters and the formation of products of the hydrolysis which cause a decreased interfacial tension.

Bartell and Miller [8] and Mack and Bartell [9] have devised improved apparatus for the measurement of interfacial tension which is based on the double capillary principle and which can be used with liquids that are either heavier or lighter than water. The later forms of their apparatus have the advantage that very small quantities of organic liquid, not to exceed 2 cc., are necessary, and even with such small quantities of liquid, precision data can be obtained.

Interfacial tension can also be obtained from surface-tension data using Antonoff's theorem, that the interfacial tension is equal to the surface tension of substance A saturated with substance B, minus the surface tension of substance B saturated with substance A, or

$$\gamma_{AB} = \gamma_A - \gamma_B \tag{108}$$

Harkins [10] states that this rule does not hold rigidly since it ignores molecular orientation and therefore the composition at the interface differs from the composition of the bulk of the liquid. He suggests that the adhesional forces residing in the different ends of the molecule at the two interfaces of the oriented molecular layer have to be taken into consideration.

Harkins and Humphrey,[11] Reynolds,[12] and Antonoff,[13-15] and

[8] Bartell, F. E., and Miller, F. L., A Method for the Measurement of Interfacial Tension of Liquid-Liquid Systems, *J. Am. Chem. Soc.*, 50: 1961–1967 (1928).

[9] Mack, G. L., and Bartell, F. E., A Double Capillary Method for the Measurement of Interfacial Tension, *J. Am. Chem. Soc.*, 54: 936–942 (1932).

[10] Harkins, W. D., The Adhesion Tension Between Mercury, Water, and Organic Substances and the Fundamental Principles of Flotation, *Proc. Natl. Acad. Sci. U. S.*, 5: 569–573 (1919).

[11] Harkins, W. D., and Humphrey, E. C., The Drop Weight Method for the Determination of Surface Tension, *J. Am. Chem. Soc.*, 38: 228–241 (1916); *cf.* also Harkins, W. D., and Humphrey, E. C., The Surface Tension at the Interface between Two Liquids, and the Effect of Acids, Salts and Bases upon Interfacial Tension, *J. Am. Chem. Soc.*, 38: 242–246 (1916).

[12] Reynolds, W. C., On Interfacial Tension. I. The Statical Measurement of Interfacial Tension in Absolute Units. II. The Relation between Interfacial and Surface Tension in Sundry Organic Solvents in Contact with Aqueous Solutions, *J. Chem. Soc.*, 119: 460–476 (1921).

[13] Antonoff, G., Über die Spannung an der Grenze zweier Flüssigkeitsschichten, *J. Russian Phys. Chem. Soc.*, 39: 342–353 (1907). Cited in *Chem. Zentr.* 1907, II, 1295.

[14] Antonow, G. N., Sur la tension superficielle à la limite de deux couches, *J. chim. phys.*, 5: 372–385 (1907).

[15] Antonoff, G. N., Interfacial Tension and Complex Molecules, *Phil. Mag.*, (VI) 36: 377–396 (1918).

Harkins and Zollman [16] likewise studied the question of the accurate measurement of interfacial tension and have presented data on various systems. Certain data of Harkins and Zollman have already been recorded in Table IV. The striking result of their work is the extremely low interfacial tension which was secured between olive oil and a 0.15 *N* solution of sodium chloride in 0.001 *N* sodium hydroxide.

Various other methods for the measurement of surface tension and interfacial tension have been used. Thus, Morgan and Stevenson [17] showed that surface tension could be calculated from the weight of a drop falling from the tip of a capillary. Harkins' values for interfacial tension were determined by a modification of this drop-weight method. Until recently it has been believed that drop-weight technics yield the most accurate value for surface tension. However Hauser *et al.*,[18] using the high-speed motion-picture camera, have shown that the form of the drop falling from a capillary tip is not as regular as has been believed and that secondary drops are formed which complicate the theoretical treatment of the phenomena. Viewed in the light of these high-speed motion pictures, the task of deriving appropriate mathematical corrections for the drop-weight surface-tension equations appears to be formidable.

Du Noüy [19] devised a torsion balance for measuring surface tension by recording the pull which is required to overcome the adherence of a ring of platinum wire to the surface of a liquid. This apparatus has been modified [20] so as to measure interfacial tensions. Green [21] combined the torsion balance principle with the drop-weight principle and devised an instrument to measure surface tension by recording the weight of a given number of drops of liquid. MacDougall [22] pointed out certain limitations of the du Noüy tensiometer. Hauser, *et al.*, likewise brings into question the accuracy of surface ten-

[16] Harkins, W. D., and Zollman, H., Interfacial Tension and Emulsification. I. The Effects of Bases, Salts, and Acids upon the Interfacial Tension between Aqueous Sodium Oleate Solutions and Benzene. II. Extremely Small Interfacial Tensions Produced by Solutes, *J. Am. Chem. Soc.*, 48: 69–80 (1926).

[17] Morgan, L. R., and Stevenson, R., The Weight of a Falling Drop and the Laws of Tate. The Determination of the Molecular Weights and Critical Temperatures of Liquids by the Aid of Drop Weights, *J. Am. Chem. Soc.*, 30: 360–376 (1908).

[18] Hauser, E. A., Edgerton, H. E., Holt, B. M., and Cox, J. T., Jr., The Application of the High-Speed Motion Picture Camera to Research on the Surface Tension of Liquids, *J. Phys. Chem.*, 40: 973–988 (1936).

[19] du Noüy, P. L., A New Apparatus for Measuring Surface Tension, *J. Gen. Physiol.*, 1: 521–524 (1919).

[20] du Noüy, P. L., An Interfacial Tensiometer for Universal Use, *J. Gen. Physiol.*, 7: 625–631 (1925).

[21] Green, R. G., The Surface Tension Balance. Apparatus for the Rapid Measurement of Surface Tension, *Ind. Eng. Chem.*, 15: 1024–1025 (1923).

[22] MacDougall, F. H., Surface Tension Determined by the Ring Method, *Science*, 62: 290–291 (1925).

sions and interfacial tensions determined with the du Noüy apparatus, since the high-speed motion camera shows that a complete film of liquid is drawn out from the surface when the ring leaves the surface.

Changes in interfacial tension are responsible for many of the phenomena characteristic of colloid systems. Emulsification appears to be primarily dependent upon interfacial tension changes. Interfacial tension changes are likewise involved in cell division. In 1876, Bütschli stated that, if a drop of oil was suspended in an aqueous solution, the droplet would break into two drops providing that a high surface tension could be produced locally at the equator of the drop. Robertson [23] contended that the droplet would pull apart at the point where the surface tension was the lowest. McClendon [24] contended that Bütschli's view was correct and carried out experiments to test the hypothesis. A chloroform-rancid olive oil droplet was suspended in a sodium chloride solution of specific gravity such that the oil droplet floated in the liquid. A dilute solution of sodium hydroxide was then allowed to flow from the tips of two pipets against the opposite poles of the oil droplet. The alkali uniting with the acid in the droplet formed a soap which decreased the surface tension at the poles of the droplet and caused the droplet to elongate into an hour-glass form or to break in two completely, owing to constriction by the higher interfacial tension at the equator.

Bancroft and Gurchot [25] repeated these experiments under somewhat more exact control and confirmed Bütschli's view. Undoubtedly interfacial tension is not the only factor operating in cell division. It does, however, provide a mechanical force which would be capable of producing the observed effects.

Surface Energy. As already noted, a liquid differs from a gas in that it has a bounding surface. The energy of a gas is solely kinetic energy. The energy of a liquid, on the other hand, is composed of two factors, the internal or kinetic energy and the surface energy. The amount of kinetic energy available depends upon the initial value of the kinetic energy per unit of mass multiplied by the weight of the material. *The surface energy of a system is the product of two quantities, the intensity factor and the capacity factor.* The *intensity factor* is the *surface tension* or the *interfacial tension.* The *capacity factor* is the *extent of surface area.* The surface energy of a system can accordingly be expressed by the equation:

$$S = \gamma s \tag{109}$$

[23] Robertson, T. B., Note on the Chemical Mechanics of Cell-Division, *Arch. Entwicklungsmech. Organ.*, 27: 29–34 (1909).

[24] McClendon, J. F., On the Dynamics of Cell Division. II. Changes in Permeability of Developing Eggs to Electrolytes, *Am. J. Physiol.*, 27: 240–275 (1910).

[25] Bancroft, W. D., and Gurchot, C., Cell Mitosis, *J. Phys. Chem.*, 31: 430–438 (1927).

where S = the surface energy;
γ = the surface tension or interfacial tension in ergs per square centimeter;
s = the surface area in square centimeters.

Surface energy is a very active form of energy and is readily converted either into work or into other forms of energy. Accordingly the free surface energy of a given system is decreased under many conditions. *A decrease in the free surface energy can be accomplished by (a) a reduction in area (reducing the capacity factor) or (b) a reduction of interfacial tension or surface tension (reducing the intensity factor).*

An example of the reduction of the surface energy by reducing the surface area is the coalescing of two mercury droplets into a single larger droplet. Such a coalescence takes place spontaneously when two clean mercury surfaces touch each other. The only way in which a pure liquid can decrease its surface energy is to decrease its surface area. Solutions, on the other hand, may have a higher or a lower surface tension than the pure solvent. In most instances, the surface tension of a solution is lower than that of the solvent, owing to the fact that a solution may decrease its surface energy by bringing into the surface area an excess of the solvent or of the solute, depending upon which one lowers the surface tension to the greatest extent.

The surface energy of a pure solvent or of a crystalloidal solution is insignificant in comparison with the kinetic energy of the system, inasmuch as a relatively insignificant amount of surface is present. On the other hand, the surface energy in a colloid system may be relatively enormous, by reason of a very large absolute surface. The ratio of surface energy to kinetic energy is expressed by the term specific surface, which is the ratio of the surface area of the disperse phase to the volume of the disperse phase,

$$\text{Specific surface} = \frac{\text{Absolute surface}}{\text{Volume}} \qquad (110)$$

Table XXV shows the changes in total surface and specific surface of a hypothetical cube, 1 cm. on an edge, which has been progressively subdivided until the particles have reached colloidal dimensions. It will be noted that the surface area has increased from 6 sq. cm. to 60 sq. m. at the upper limit of the colloidal realm, and to 6000 sq. m. for a particle 1 mμ in diameter. The specific surface has increased from 6 to 60,000,000. During this great increase in specific surface, the internal energy or kinetic energy has remained constant. It is usually agreed that if the specific surface (s/V) is less than 10,000, then the internal energy of a system predominates. On the other hand, if s/V is greater than 10,000, then the surface energy becomes the pre-

TABLE XXV

SURFACE AREA POSSIBLE BY THE SUBDIVISION TO COLLOIDAL DIMENSIONS OF A CUBE 1 CM. ON AN EDGE

Edge of Cube	Number of Cubes	Total Surface	Relative Surface Energy,* Assuming That Water Is Being Subdivided, Ergs
1 cm.	1	6 sq. cm.	438
0.1 μ	1,000,000,000,000,000	60 sq. m. (645.84 sq. ft.)	43,800,000
0.01 μ	1,000,000,000,000,000,000	600 sq. m. (6458.4 sq. ft.)	438,000,000
1 mμ	1,000,000,000,000,000,000,000	6000 sq. m. (64,584 sq. ft.)	4,380,000,000 (105 cal.)

* The energy in this column is the calculated surface energy due to increased surface. No account has been taken of other possible forms of energy, such as electrical force, etc.

dominating form of energy, and when the specific surface reaches such enormous values as are reached within the colloid realm, the reactions which take place are to a large measure reactions involving surface energy changes. For this reason many substances which are totally different in their chemical composition acquire properties, when in the colloid state, which are similar or identical.

Every colloidal particle is surrounded by a film of the dispersions medium. Accordingly we have operating in these films an interfacial tension which controls, at least in a large measure, the energy relationships of a liquid-solid or a liquid-liquid interface in the same way that the surface tension expresses the energy relationships at a gas-liquid interface. Although we can measure interfacial tension at a liquid-liquid interface, we are as yet unable to measure directly the forces of interfacial tension at a solid-liquid or a solid-gas interface. It is highly probable, however, that these forces are as great as, if not greater than, the known forces operating at liquid-liquid or liquid-gas interfaces. Even if we postulate forces no greater than those of liquid-gas interfaces, we have forces of sufficient magnitude to explain the surface reactions characteristic of colloid systems.

Wo. Ostwald [26] ascribes to Wenzel the law that *"the amount of chemical change in a unit of time is proportional to the absolute surface."* Since colloid systems show a great absolute surface, they also show marked chemical activity.

Powdered sulfur is practically without action on a silver surface; colloidal sulfur, on the other hand, has an energetic action on a silver surface, causing the formation of the black silver sulfide. A smooth

[26] Ostwald, Wo., A Handbook of Colloid-Chemistry, translated by Martin H. Fischer, p. 93, P. Blakiston's Son and Company, Philadelphia (1915).

polished platinum sheet can be inserted into a solution of hydrogen peroxide without causing any appreciable amount of decomposition of the peroxide; if the platinum foil is roughened, a slow evolution of oxygen takes place; if powdered platinum is added to the hydrogen peroxide, the oxygen is formed rather rapidly; platinum black causes an energetic decomposition; and a colloidal platinum sol added to hydrogen peroxide may cause a violent explosion. Colloidal platinum in a dilution as great as 1 gram atom of platinum in 70,000,000 liters of water can be detected by its decomposing action of hydrogen peroxide. No change in the chemical nature of the platinum has taken place, but the change in specific surface has shifted the chemical equilibrium in the same way that a rise in temperature would have shifted it. Bancroft and Magoffin [27] note that a number of reactions are affected by the nature of the cathode surface. Thus, at 25° there is practically no reduction of chlorate to chloride when a smooth platinum cathode is used, but reduction takes place readily when a pulverulent platinum or copper cathode is used. Similarly NO_3 and NO_2 are reduced about equally readily with a smooth platinum cathode, but NO_2 is reduced more readily than NO_3 by a platinized platinum cathode, and NO_3 is reduced more readily than NO_2 by pulverulent copper or silver cathodes. Bancroft suggests that these results may be caused by an adsorption and activation which take place prior to reduction. It is entirely probable that many of the reactions which we call catalytic are in reality due to a change in the specific area of the substance used as the catalyst. In fact, this may well be the predominating factor.

In a reaction taking place in a heterogeneous system, the tension at the interface is either increased or decreased according to the effect of the reacting product on interfacial tension. If the interfacial tension is increased, less energy is available for the chemical reaction and the reaction slows up. If the interfacial tension is decreased, more energy is available for the chemical reaction and the equilibrium is shifted toward an increased reaction. If salts of the fatty acids are brought into contact with a large surface area, increased hydrolysis takes place and an excess of fatty acids is concentrated in the interface with the excess of base in the liquid. This causes a disturbance in the equilibrium, shifting it to the right; consequently more of the fatty acid salt must be hydrolyzed to establish a new equilibrium:

$$MAc + H_2O \rightleftarrows MOH + HAc$$

Powdering a substance before dissolving it replaces work which must be expended in the solution process, by mechanical work. Consequently more energy is available for the work of solution of a small

[27] Bancroft, W. D., and Magoffin, J. E., Energy Levels in Electrochemistry, *J. Am. Chem. Soc.*, 57: 2561–2565 (1935).

particle than of a large particle. Accordingly, finely powdered substances show a greater solubility and a greater heat of solution than do coarsely powdered substances or large crystals. Stas, in 1870, obtained the following values as the solubility of silver chloride: granular AgCl, 0.001 gram per liter at 15°; powdered AgCl, 0.0060 gram per liter at 17°; flocculent AgCl, 0.0140 gram per liter at 20°.

Hulett [28-30] in a study of the solubility of gypsum found that particles 0.3 μ in diameter had a solubility of 18.2 millimoles per liter, whereas particles 2 μ in diameter showed the normal solubility of 15.3 millimoles per liter.

Hulett, similarly, found that small particles of barium sulfate of 0.1 μ radius had a solubility of 4.15 millimoles per liter, whereas particles of 1.8 μ radius had the normal solubility of 2.29 millimoles.

Hulett calculated from his data the interfacial tension of the solid-liquid. However, Freundlich [31] points out that the equation which Hulett used is incorrect. In its place, he proposes the equation

$$\frac{RT}{M} \log_e \frac{C_2}{C_1} = \frac{2\gamma_{SL}}{rD} \tag{111}$$

or

$$\gamma_{SL} = \frac{RTDr}{2M} \log_e \frac{C_2}{C_1} \tag{112}$$

where R = the gas constant = 8.31×10^7 ergs;
T = the absolute temperature;
D = the density of the solid phase;
r = the radius of the smaller particles;
C_2 = the concentration of the solution in equilibrium with the particles of radius, r;
C_1 = the concentration of the solution in equilibrium with the larger particles, *i.e.*, the concentration of a solution in equilibrium with the massive solid phase;
γ_{SL} = the interfacial tension of the solid-liquid;
M = the molecular weight of the substance the solubility of which is being determined.

Using equation (112) and Hulett's data, the interfacial tension of gypsum-water is found to be 1,140 ergs per square centimeter, and of the barium sulfate-water system, 1,420 ergs per square centimeter.

[28] Hulett, G. A., Beziehungen zwischen Oberfläschenspannung und Löslichkeit, *Z. physik. Chem.*, 37: 385–406 (1901).

[29] Hulett, G. A., Löslichkeit und Korngrösse. Erwiderung an Herrn Prof. F. Kohlrausch, *Z. physik. Chem.*, 47: 357–367 (1904).

[30] Hulett, G. A., Solubility and Size of Particles, Chap. 36, Colloid Chemistry, Vol. I, edited by J. Alexander, Chemical Catalog Company, New York (1926).

[31] Freundlich, H., Kapillarchemie, p. 144, Leipzig (1909).

Jones [32] later proposed a more complicated equation, taking into consideration the dissociation of the salts in the solution. His equation yields 1,048 ergs per square centimeter for the gypsum system and 1,332 ergs per square centimeter for the barium sulfate system.

Dundon and Mack,[33] and Dundon [34] repeated Hulett's experiments and extended them to other salts. They point out that Hulett's values for gypsum are too high, probably because during the powdering of the gypsum a part of the crystal water was lost and $CaSO_4 \cdot H_2O$ has a much greater solubility than gypsum.

TABLE XXVI

INTERFACIAL TENSIONS AT SOLID-WATER INTERFACES

Substance	Diameter of Particle, μ	Increase in Solubility, Per Cent	γ
PbI_2	0.4	2.0	130
$CaSO_4 \cdot 2H_2O$	0.5	4.8	356
$CaSO_4 \cdot 2H_2O$	0.2–0.3	12.3	385
Ag_2CrO_4	0.3	10.0	575
PbF_2	0.3	9.0	900
$SrSO_4$	0.25	26.0	1400
$BaSO_4$	0.1	80.0	1250

Table XXVI gives their data. The last column in the table represents as nearly as can be determined with our present technic the probable interfacial tensions in ergs per square centimeter of the various substances against a water interface. Glasstone [35] from similar studies calculated an interfacial energy between lead oxide and sodium hydroxide of 1,860 ergs per square centimeter at 20°

Lipsett, Johnson, and Maass [36] found a difference of 24.0–40.7 calories per mole in the heat of solution of finely divided sodium chloride where the particles had an average diameter of 1.2–1.4 μ, over that for the heat of solution of the coarsely crystalline salt. They point out

[32] Jones, W. J., Über die Grösse der Oberfläschenergie fester Stoffe, *Z. physik. Chem.*, 82: 448–456 (1913).

[33] Dundon, M. L., and Mack, E., Jr., The Solubility and Surface Energy of Calcium Sulfate, *J. Am. Chem. Soc.*, 45: 2479–2485 (1923).

[34] Dundon, M. L., Surface Energy of Several Salts, *J. Am. Chem. Soc.*, 45: 2658–2666 (1923).

[35] Glasstone, S., Physical Chemistry of the Oxides of Lead. Part I. The solubility of Lead Monoxide, *J. Chem. Soc.*, 119: 1689–1697 (1921).

[36] Lipsett, S. G., Johnson, F. M. G., and Maass, O., The Surface Energy and the Heat of Solution of Solid Sodium Chloride. I and II. *J. Am. Chem. Soc.*, 49: 925–943; 1940–1949 (1927); III, *ibid.*, 50: 2701–2703 (1928).

that the difference in heat of solution is the heat which is bound up in the excess surface energy of the fine salt, and from their experimental values they find the surface energy of sodium chloride to range from 356 to 406 ergs per square centimeter.

We have already noted that colloidal particles may increase in size, as the result of crystal growth (*cf.* equation [3]), and that the rate of crystal growth depends in part upon the supersaturation of the solution. It must be obvious that, if small particles have a greater solubility than large particles and if a substance dispersed in small particles has a greater specific surface and consequently a greater surface energy per unit of mass, small particles will dissolve in a solution which has not yet reached the saturation value for such particles but which is already supersaturated with respect to large crystals or a plane surface. Accordingly in a system containing particles of various sizes, equilibrium will eventually be established when all small particles have disappeared and only relatively large crystals are present.

Many papers have been written to present experimental work involving these considerations of crystal growth. Lipsett, Johnson, and Maass note that, when their finely divided salt was placed in a moist atmosphere, water was rapidly absorbed, 1 gram of the salt absorbing 1 mg. of water in 20 minutes, 3 mg. in 2 hours, and 6.9 mg. in 5 hours. The 1 gram of salt originally occupied a volume of 8 cc. At the end of 5 hours the volume had contracted to approximately one-fourth of its original bulk. A microscopical examination showed that, whereas the original dry salt had contained innumerable particles. approximately 1 μ in diameter, after 30 minutes the main bulk of the salt was made up of particles ranging from 4 μ to 10 μ in diameter. They ascribe this change to the greater solubility of the small particles which dissolved in the water that was absorbed and then recrystallized as larger particles, and note that a few milligrams of water will act catalytically in transforming a large mass of finely divided salt into salt of much coarser degree of subdivision.

Kolthoff [37] considered the solubility of large and small crystals as a factor in analytical procedures, pointing out that with hard crystals, such as $BaSO_4$, the *relative* supersaturation may be enormous ($\pm$ 1,000) when large crystals and small crystals are compared, whereas with soft crystals, such as Ag_2CrO_4 (hardness *ca.* 2), the ratio value is only about 4.0 and with PbI_2 (hardness < 2) the ratio is only 1.38. In the latter substances the ratio is not great enough to make appreciable differences in analytical determinations, although for nephelometric analyses it would still be a factor. Grosvenor [38] ob-

[37] Kolthoff, I. M., Über die Empfindlichkeit chemischer Fällungsreaktionen, *Z. anal. Chem.*, 86: 34–40 (1931).

[38] Grosvenor, W. M., A Surface Tension Effect, *Science*, 72: 244–245 (1930).

served air droplets in Nujol ($\gamma = 44$ dynes). The air droplets disappeared rapidly after they reached a diameter of about 5 μ. At about 3 μ the shrinkage was rapid enough to be followed continuously, and at 1.5 μ they disappeared in less than 0.5 second. At 5 μ the pressure within the droplet, due to surface tension, was about 8 pounds per square inch above atmospheric pressure, and at 1.5 μ the pressure had risen to about 2 atmospheres. The collapse was due to the solution of the small bubble in the oil, larger bubbles growing at the expense of the smaller ones. Grosvenor states that bubbles less than 3 μ in diameter cannot be stable in the liquid and they will vanish by loss of gas either to the larger bubbles or to the air-liquid surface. Many highly viscous liquids, such as syrups and lacquers, become freed from air bubbles by this differential solution-gas liberation process.

If finely divided materials possess an excess surface energy, they should melt at a lower temperature than larger particles. Here, again, we have the principle of mechanical work expended in subdividing the particle, replacing thermal work. Palow [39, 40] observed that increasing specific surface of salol (phenyl salicylate) one hundred times depresses the melting point 2.8° C. This may explain why chemists do not always agree on the exact melting point of a chemical compound.

This phenomenon may also explain in part why certain biological organisms are not frozen when the temperature falls to a few tenths of a degree below the freezing point, or why water in fine capillary spaces, such as the interstices of a clay soil, has a freezing point below 0° C. If water is finely divided, it should cause a depression of the temperature at which ice forms. Parker [41] investigated the effect of mixing finely divided materials with water, benzene, and nitrobenzene. In each instance, he used materials which are insoluble in the liquid phase, so that the depression of the freezing point which was observed cannot be ascribed to the presence of a solution. It must be obvious from Table XXVII that it is impossible to calculate the concentration of a solute in a solvent from freezing-point data, if inert materials possessing large specific surface are present in the system at the time of making the freezing point measurements.

REINDERS' THEOREM.—Reinders [42] investigated the distribution of a suspended powder or of a colloidally dispersed material between two

[39] Palow, P., Über die Abhängigkeit des Schmelzpunktes von der Obenfläschenenergie eines festen Körpers, *Z. physik. Chem.*, 65: 1–35; 545–548 (1908–9).

[40] Palow, P., Über die Schmelztemperatur der Körner des Salols, *Z. physik. Chem.*, 74: 562–566 (1910).

[41] Parker, F. W., The Effect of Finely Divided Material on the Freezing Points of Water, Benzene, and Nitrobenzene, *J. Am. Chem. Soc.*, 43: 1011–1018 (1921).

[42] Reinders, W. von, Die Verteilung eines suspendierten Pulvers oder eines kolloid gelösten Stoffes zwischen zwei Lösungsmitteln, *Kolloid Z.*, 13: 235–241 (1913).

mutually insoluble liquids. Let us assume, for example, that we have a red gold hydrosol, and this is mixed with petroleum ether. Will the disperse phase remain in the water or will it migrate to the petroleum ether, or will it form a layer at the interface between the water and the petroleum ether? The distribution will depend upon three interfacial tensions, the interfacial tension between solid and water, γ_{sw}; the interfacial tension between water and petroleum ether, γ_{wo}; and the interfacial tension between solid and petroleum ether, γ_{so}.

TABLE XXVII

CHANGE IN THE FREEZING POINT OF LIQUIDS WHEN IN THE FORM OF FILMS ON FINELY DIVIDED POWDERS (DATA OF PARKER)

Substance	Liquid Added, per cent by weight	Depression of Freezing Point over That of Pure Liquid, ° C.	Substance	Liquid Added, per cent by weight	Depression of Freezing Point over That of Pure Liquid, ° C.
	Water			Benzene	
Al_2O_3.....	25.0	2.118	SiO_2.....	3.3	0.670
Al_2O_3.....	30.0	1.227	SiO_2.....	5.0	0.490
Al_2O_3.....	35.0	0.650	SiO_2.....	10.0	0.225
Al_2O_3.....	40.0	0.370	SiO_2.....	20.0	0.110
	Benzene			Nitrobenzene	
Al_2O_3.....	30.0	1.337	Al_2O_3.....	50.0	1.720
Al_2O_3.....	35.0	0.682	Al_2O_3.....	60.0	1.175
Al_2O_3.....	40.0	0.492	Al_2O_3.....	70.0	0.810
Al_2O_3.....	50.0	0.212	Al_2O_3.....	80.0	0.580
			Al_2O_3.....	100.0	0.200

If $\gamma_{so} > \gamma_{wo} + \gamma_{sw}$, the solid will remain suspended in the water.

If $\gamma_{sw} > \gamma_{wo} + \gamma_{so}$, the solid will leave the water and go into the oil phase.

If $\gamma_{wo} > \gamma_{sw} + \gamma_{so}$, or if none of the three interfacial tensions is greater than the sum of the other two, the solid particles will collect at the boundary between the water and the oil.

These conditions will also hold for a particle which comes in contact with a film of oil instead of a layer of oil or a globule of oil, and will determine whether or not the particle is wetted by the oil film. It will be wetted, if $\gamma_{sw} >$ the other two interfacial tensions. It will remain in contact with the oil film, if $\gamma_{wo} >$ the other two interfacial tensions. It will not be wetted, if $\gamma_{so} >$ the other two interfacial ten-

sions. These principles apply to practical problems and are particularly important in dealing with the adherence of insecticidal and fungicidal dusts and oil sprays.

CONTACT ANGLES, ADHESION TENSION, AND DEGREE OF WETTING.—Bartell [43-55] and his students during recent years have contributed greatly to our knowledge of adhesion tension and technics by which we can express quantitatively the degree of wetting. His entire series of papers should be consulted by the student who is interested in the energy relationships of solid-liquid interfaces.

Contact Angles.—If a drop of liquid is placed in contact with a solid, the resulting equilibrium will be determined by three factors—the surface tension of the liquid, the surface tension of the solid, and the solid-liquid interfacial tension. We can see from Reinders' theorem that if the surface tension of the solid is greater than the sum

[43] Bartell, F. E., and Osterhof, H. J., Determination of the Wettability of a Solid by a Liquid, *Ind. Eng. Chem.*, 19: 1277–1280 (1927).

[44] Bartell, F. E., and Osterhof, H. J., The Measurement of Adhesion Tension Solid Against Liquid, Colloid Symposium Monograph, Vol., V, pp. 113–134, Chemical Catalog Company, New York (1928).

[45] Bartell, F. E., and Miller, F. L., Degree of Wetting of Silica by Crude Petroleum Oils, *Ind. Eng. Chem.*, 20: 738–742 (1928).

[46] Bartell, F. E., and Sloan, C. K., An Interferometric Investigation of Adsorption by Pure Carbon from Non-Aqueous Binary Systems, *J. Am. Chem. Soc.*, 51: 1637–1643 (1929); Adsorption by Pure Carbon from Non-Aqueous Binary Systems over the Entire Concentration Range, *ibid.*, 1643–1656 (1929).

[47] Bartell, F. E., and Smith, C. N., Adhesion Tension Values of Different Types of Carbon Black Against Water and Against Benzene, *Ind. Eng. Chem.*, 21: 1102–1106 (1929).

[48] Osterhof, H. J., and Bartell, F. E., Three Fundamental Types of Wetting. Adhesion Tension as the Measure of Degree of Wetting., *J. Phys. Chem.*, 34: 1399–1411 (1930).

[49] Bartell, F. E., Wetting of Solids by Liquids, in Alexander's Colloid Chemistry, Vol. III, pp. 41–60, Chemical Catalog Company, New York (1931).

[50] Davis, N. S., Jr., and Curtis, H. A., Preferential Wetting of Solids by Liquids, *Ind. Eng. Chem.*, 24: 1137–1140 (1932).

[51] Bartell, F. E., and Jennings, H. Y., Adhesion Tension of Liquids Against Strongly Hydrophilic Solids, *J. Phys. Chem.*, 38: 495–501 (1934).

[52] Bartell, F. E., and Walton, C. W., Jr., Alteration of the Surface Properties of Stibnite as Revealed by Adhesion Tension Studies, *J. Phys. Chem.*, 38: 503–511 (1934).

[53] Bartell, F. E., and Hatch, G. B., The Wetting Characteristics of Galena, *J. Phys. Chem.*, 39: 11–23 (1935).

[54] Bartell, F. E., Culbertson, J. L., and Miller, M. A., Alteration of the Free Surface Energy of Solids. I. Vertical-Rod Method for the Measurement of Contact Angles and Preliminary Study of Effect of Heat Treatment on Magnitude of Contact Angles, *J. Phys. Chem.*, 40: 881–888 (1936).

[55] Bartell, F. E., and Miller, M. A., Alteration of the Free Surface Energy of Solids. II. Effect of Heat Treatment of Metals in Air, *J. Phys. Chem.*, 40: 889–894 (1936); III. Effect of Heat Treatment of Metals in a Vacuum and in Several Gases, *ibid.*, 895–904 (1936).

of the two tensions, the drop will spread and become a film upon the solid. If, however, the surface tension of the solid is less than the sum of the other two tensions, then the liquid drop will remain upon the solid and there will be a definite angle of contact between the liquid and the solid which will be related to the adhesion tension between the liquid and the solid and also to the degree that the liquid wets the solid. If the angle of contact is zero, there will be complete wettability and a high adhesion tension. If the angle of contact is 180 degrees, no wetting of the solid by the liquid occurs. Figure 67 shows contact angles of different magnitudes. Where the liquid actually contacts the solid, there is a decrease in free surface energy, and the following relationships must hold:

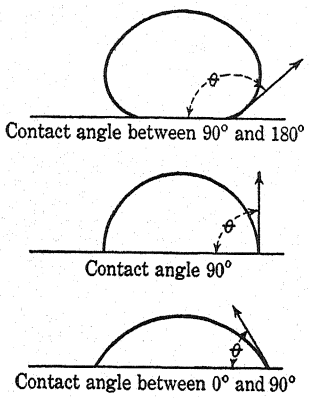


FIG. 67.—Types of liquid-solid contact angles as indicative of different degrees of wettability.

$$\gamma_s + \gamma_l = \gamma_{sl} + \Delta F \qquad (113)$$

where ΔF = the decrease in free surface energy.

Young, in 1805, derived the equation for the equilibrium conditions existing in a solid-liquid system in terms of the contact angle and the interfacial tension. His equation is

$$\gamma_s - \gamma_{sl} = \gamma_l \cos \theta \qquad (114)$$

where θ = the contact angle.

Contact angles can be measured by placing a drop of a liquid upon a plane plate of the solid material and viewing the drop through a microscope with the objective horizontal to the plane of contact, or the drop on the plane surface can be placed in the beam of a projection lens (the optical system of the Zsigmondy slit ultramicroscope is well adapted to such use), and the enlarged image of the drop in contact with the plane surface can be thrown upon a ground-glass screen where the outline of the contact angle can be drawn and later measured.

Bartell has developed methods for the determination of the contact angle by the pressure which is necessary to displace one liquid by another, and this method has been found of great value in certain industrial problems. Unfortunately it is necessary to know the average pore radius in the diaphragm in which the liquid displacement occurs, and no method has yet been devised for using this method in diaphragms of hydrophilic (or lyophilic) colloids where the pore radius is different in one liquid from that in another liquid.

Bartell has likewise devised methods of measuring the contact angle by dipping a vertical rod of the solid to be tested into a liquid and throwing the image of the angle either on the screen where it can be measured or on a photographic plate where it can be permanently recorded. With the vertical-rod method the advancing contact angle can be measured by slowly lowering the rod into the liquid or the receding angle can be measured by slowly raising the rod from the liquid.

Adhesion Tension.—Freundlich states that the adhesion tension between a solid and a liquid is a fraction of the surface tension of the liquid and equals the term ($\gamma_l \cos \theta$) in equation (114).

$$A_{1,2} = \gamma_l \cos \theta \tag{115}$$

where $A_{1,2}$ = the adhesion tension of solid-liquid.

Then from equation (114), we have

$$A_{1,2} = \gamma_s - \gamma_{sl} \tag{116}$$

The adhesion tension, therefore, is the difference in dynes per centimeter or ergs per square centimeter between the surface tension of the solid measured against air and its interfacial tension against the liquid. It is the decrease in free surface energy which occurs when 1 sq. cm. of solid-liquid interface is substituted for 1 sq. cm. of solid-air interface, and accordingly is the energy factor upon the magnitude of which depends the wetting or non-wetting of a solid by a liquid.

Bartell and Osterhof, using the displacement method, determined adhesion tensions at silica-liquid and carbon black-liquid interfaces. Table XXVIII shows certain of their data. It is obvious that a sus-

TABLE XXVIII

SHOWING ADHESION TENSIONS OF SILICA AND CARBON BLACK FOR VARIOUS LIQUIDS (DATA OF BARTELL AND OSTERHOF)

Liquid	Silica-Liquid Interface		Carbon Black-Liquid Interface	
	Displacement Pressure, dynes/cm.	Adhesion Tension, dynes/cm.	Displacement Pressure, dynes/cm.	Adhesion Tension, dynes/cm.
Water	.....	82.82	.....	54.74
Aniline	8	82.00	1266	60.51
Carbon tetrachloride	409	40.69	6935	89.45
Hexane	395	42.13	3330	69.93
Benzene	295	52.43	5775	81.08
α-Brom naphthalene	397	41.92		

pension will be most stable in that liquid which has the highest adhesion tension for the solid. Thus, from Table XXVIII it is evident that SiO_2 will form the most stable dispersion in water and carbon black will be most stable in carbon tetrachloride. These discussions of adhesion tensions are simply restating in other terms the factors which we have already noted under Reinders' theorem.

Since immersion in a particular liquid involves wettability, solid-liquid adhesion tensions become of great importance in the adherence of insecticides, dusts, oil sprays, paints, varnishes, etc. Bartell and Walton (*loc. cit.*) essentially propose adhesion tensions as a quantita-

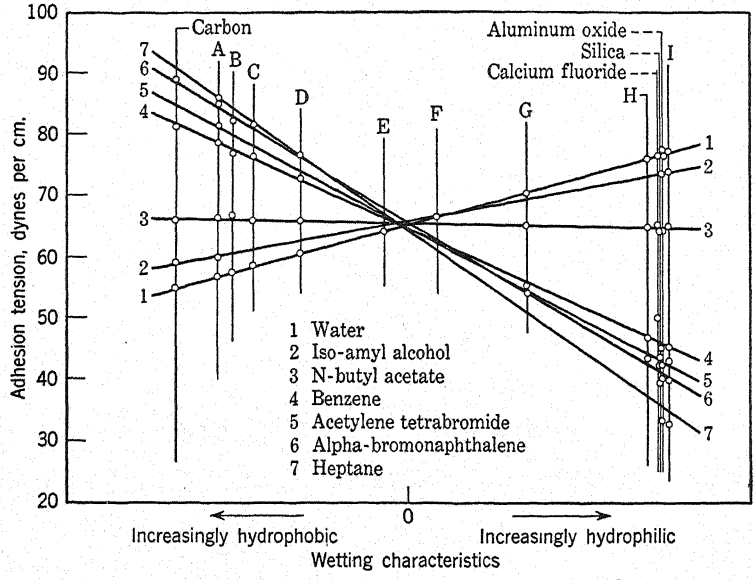


FIG. 68.—Showing the relationships between adhesion tension, the polar or non-polar nature of the liquid, and the hydrophilic or hydrophobic nature of the solid. (Data of Bartell and Walton, by permission, *Journal of Physical Chemistry*.)

tive measure of the hydrophilic or hydrophobic properties of surfaces, *i.e.*, wettability by water or wettability by oils with Al_2O_3 and carbon as solid extremes, and water and *n*-heptane as extreme liquids. They were able to prepare by heat treatment samples of stibnite (Sb_2S_3) varying from extreme wettability by water to extreme wettability by oil. Table XXIX shows certain of their adhesion tension data. Figure 68 shows the adhesion tensions of the various stibnites noted in Table XXIX and of various other solids as measured against a variety of liquids. Rather interestingly, *n*-butyl acetate appears to show no preferential wetting in favor of any of these solids. Liquids more polar than *n*-butyl acetate tend to preferentially wet alumina and silica.

TABLE XXIX

ADHESION TENSIONS OF HEAT-TREATED STIBNITE AGAINST WATER AND BENZENE
(Data of Bartell and Walton)

Stibnite	Treatment	A_{sl} (H_2O), dynes/cm.	A_{sl} (C_6H_6), dynes/cm.
A	Ground only	56.5	78.4
B	Heat 1 hr. at 170°	57.2	76.6
C	Heat 2 hr. at 170°	58.1	76.0
D	Heat 3 hr. at 170°	60.3	72.6
E	Heat 4 hr. at 170°	64.2	....
F	Heat 5 hr. at 170°	66.5	....
G	Heat 6 hr. at 170°	70.5	55.0
H	Heat 8 hr. at 170°	76.0	47.0
I	Heat 8 hr. at 400–420° (in nitrogen)	77.1	45.4

Liquids less polar tend to preferentially wet carbon. As Bartell suggests, studies of this sort may give means of quantitatively evaluating degrees of hydrophilic or hydrophobic characteristics.

Mudd and Mudd [56] suggested a technic whereby one could study the relative wettability of particles by oil or by water. The particles suspended in water were placed on a microscope slide and a drop of oil was added adjacent to the water drop. When a cover slip was placed over this preparation, an oil-water boundary was formed; and when the slide was observed under a microscope (preferably with dark-field illlumination), it was observed that the oil boundary advanced across the field. When the oil boundary reached a particle which was easily wetted by oil, the particle progressed through the oil-water interface with little or no distortion of the interface. If, however, the particle was not wetted by oil or was very difficultly wetted, the interface was compressed and distorted, although eventually the particle might break through and be engulfed by the oil. In extreme cases such engulfment left the particle with a surrounding water film so that in reality there was no wetting of the particle by the oil.

Nugent [57] diagrammed the appearance of the oil-water interface of the Mudd and Mudd phenomenon in eight degrees of wettability from an extreme case where a particle "dissolves" in the oil to one

[56] Mudd, S., and Mudd, E. B. H., The Penetration of Bacteria Through Capillary Spaces. IV. A Kinetic Mechanism in Interfaces, *J. Exptl. Med.*, 40: 633–645 (1924); Certain Interfacial Tension Relations and the Behavior of Bacteria in Films, *ibid.*, 40: 647–660 (1924).

[57] Nugent, R. L., The Application of the Mudd Interfacial Technique in the Study of Protective Protein Films in Oil-in-Water Emulsions, *J. Phys. Chem.*, 36: 449–466 (1932).

where a particle passes the interface only when surrounded by a water film or where the moving boundary simply pushes the particle ahead of it. Nugent's diagram is shown in Fig. 69. Moyer [58] used this technic to determine the physical state of the surface of latex particles from various species of *Euphorbia* and found that those species with a low isoelectric point (*vide supra*) of $p\mathrm{H} \pm 3.0$ showed preferential wettability by oil, whereas those with a higher isoelectric point of $p\mathrm{H} \pm 4.5$–5.0 possessed surfaces which were resistant to oil wetting and were preferentially wetted by water. These observations confirmed the electrokinetic studies where the assumption had been made that the former probably had a sterol-like surface, whereas the latter possessed a protein protective surface.

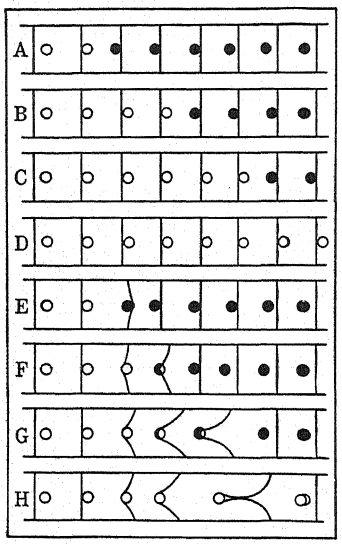


FIG. 69.—A diagrammatic representation of various degrees of wettability, as shown by the Mudd and Mudd technic. (After Nugent.)

THE ORIENTATION OF MOLECULES IN SURFACE OR INTERFACIAL FILMS.—We have already referred casually to the work of Hardy, Harkins, Langmuir, and Adam, as having shown that whereas the molecules in the body of a liquid are apparently distributed at random, those molecules which enter into the surface film are in general arranged in a more or less orderly fashion. Although undoubtedly the germ of the idea of molecular orientation originated with Hardy,[59, 60] nevertheless Harkins,[61–65] and Langmuir,[66] independently developed this idea

[58] Moyer, L. S., On the Surface Composition of Certain Latex Particles, *Am. J. Botany*, 22: 609–627 (1935).

[59] Hardy, W. B., The Tension of Composite Fluid Surfaces and the Mechanical Stability of Films of Fluid, *Proc. Roy. Soc. (London)*, (A) 86: 610–635 (1912).

[60] Hardy, W. B., The Influence of Chemical Constitution upon Interfacial Tension, *Proc. Roy. Soc. (London)*, (A), 88: 303–313 (1913); The Tension of Composite Fluid Surfaces. II., *Proc. Roy. Soc. (London)*, (A), 88: 313–333 (1913).

[61] Harkins, W. D., Brown, F. E., and Davies, E. C. H., The Structure of the Surfaces of Liquids, and Solubility as Related to the Work Done by the Attraction of Two Liquid Surfaces as They Approach Each Other. V. Surface Tension, *J. Am. Chem. Soc.*, 39: 354–364 (1917).

[62] Harkins, W. D., Davies, E. C. H., and Clark, G. L., The Orientation of Molecules in Surfaces of Liquids, the Energy Relations at Surfaces, Solubility, Adsorption, Emulsification, Molecular Association, and the Effect of Acids and Bases on Interfacial Tension. Surface Energy VI., *J. Am. Chem. Soc.*, 39: 541–596 (1917).

[63] Harkins, W. D., Clark, G. L., and Roberts, L. E., The Orientation of Mole-

along somewhat different lines. The work of Harkins originated from his interest in the subject of energy at interfaces between liquids; that of Langmuir originated from his work on liquid films. Both have come to the general conclusion that surface tension or interfacial tension phenomena are, in general, characterized by the orientation and the packing of molecules in surface layers and that the forces involved in this action are the forces of solution and the forces of interfacial tension. Hardy [67, 68] extended his work in a different direction in studying the problems of friction and lubrication.

In reality, monomolecular films existing at interfaces constitute a different state of matter from that which we are accustomed to consider, for they are *two-dimensional gases, liquids, and solids.*[69] Since they have length and breadth but only monomolecular depth, it is evident that they can move only in two directions and not in a third direction. In a highly expanded form they behave as two-dimensional gases; when the molecules come closer together so as to approximate the distances between molecules in the liquid state, they behave as two-dimensional liquids; and when still further compressed they become two-dimensional solids. The equations of state for gases, liquids, and solids accordingly have to be modified to account for the kinetic behavior of the molecules in these two-dimensional films.

Concepts introduced by studies on molecular orientation may be summarized as follows.[70]

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cules in Surfaces, Surface Energy, Adsorption, and Surface Catalysis. V. The Adhesional Work between Organic Liquids and Water, *J. Am. Chem. Soc.*, 42: 700–712 (1920).

[64] Harkins, W. D., and Cheng, Y. C., The Orientation of Molecules in Surfaces. VI. Cohesion, Adhesion, Tensile Strength, Tensile Energy, Negative Surface Energy, Interfacial Tension, and Molecular Attraction, *J. Am. Chem. Soc.*, 43: 35–53 (1921).

[65] Harkins, W. D., and Beeman, N., Emulsions: Stability, Area per Molecule in the Interfacial Film, Distribution of Sizes and the Oriented Wedge Theory, *J. Am. Chem. Soc.*, 51: 1674–1694 (1929); (*Cf.* also Harkins' papers in Alexander's Colloid Chemistry, pp. 192–264, and Colloid Symposium Monograph, Vol. II, pp. 141–173.)

[66] Langmuir, I., The Constitution and Fundamental Properties of Solids and Liquids. II. Liquids, *J. Am. Chem. Soc.*, 39: 1848–1906 (1917). (*Cf.* also Colloid Symposium Monograph, Vol. III, pp. 48–75.)

[67] Hardy, W. B., Friction, Surface Energy and Lubrication, Chap. XIII of Colloid Chemistry, Theoretical and Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, New York (1926).

[68] Hardy, W. B., Problems of the Boundary State, *Phil. Trans. Roy. Soc. (London)*, (A), 230: 1–37 (1931).

[69] Langmuir, I., Two-Dimensional Gases, Liquids and Solids, *Science*, 84: 379–383 (1936).

[70] In addition to cited reference see Rideal, E. K., An Introduction to Surface Chemistry, Second Edition, University Press, Cambridge, England (1930); Adam, N. K., The Physics and Chemistry of Surfaces, Oxford University Press (1930).

Liquids may be divided, in general, into two great classes, those in which the molecule is essentially symmetrical and those in which the molecule is more or less unsymmetrical. Pentane may be taken as an example of a symmetrical molecule, and acetic acid as an example of an unsymmetrical molecule. In the pentane molecule the two ends of the hydrocarbon chain are identical. Accordingly we would expect the two ends to behave identically toward an interface. In the case of acetic acid the two ends of the molecule are very dissimilar, one being of hydrocarbon nature, the other (—OH) very similar to water. Accordingly we would expect the two ends of the acetic acid molecule to behave differently toward an interface. To express these differences, the term *polar group* has been introduced, and the radicals —OH, —COOH, —CHO, —CN, —$CONH_2$, —SH, —NH_2, —$NHCH_3$, —NCS, —COR, —COOM, —COOR, —NO_2, —$CH = CH_2$, —$C{\equiv}CH$, and groups which contain oxygen, nitrogen, sulfur, iodine, bromine, and chlorine, and double- and triple-bonds have been called polar groups, and compounds containing these groups have been designated as *polar compounds*.

A polar group confers upon an organic compound a certain solubility in water. Thus, we have in methane a gas which is relatively non-water-soluble. When we introduce a polar group, as in methyl alcohol, methyl amine, etc., we form a compound which is water-soluble. One portion (CH_3—) of a molecule of methyl alcohol is of hydrocarbon nature; the other portion (—OH) is closely allied to water, and as such shows an affinity for water. If then a small amount of methyl alcohol is dissolved in water, it will concentrate in the surface film, on account of the fact that methyl alcohol lowers the surface energy at an air-water interface. The fact that the CH_3— group retains a part of the properties of a hydrocarbon and the —OH group retains a part of the properties of water causes the methyl alcohol molecules to orient themselves in the interfacial film, with the hydrocarbon chain toward the vapor phase and the —OH group toward the water phase. As we lengthen the hydrocarbon chain in passing to ethyl alcohol, propyl alcohol, butyl alcohol, amyl alcohol, etc., there is a progressive intensification of the hydrocarbon properties residing in the molecule, and a corresponding lessening in the similarity of the molecule to a water molecule. Accordingly the solubility of the alcohols in water decreases as the carbon chain is lengthened, and correspondingly their solubility in organic solvents increases.

In the case of acids, when we approach lauric acid, we reach a point where the interface between an aqueous solution of lauric acid and air is essentially a hydrocarbon interface.[71] Figure 6 has already been

[71] The point at which the liquid-air interface becomes essentially a hydrocarbon-air interface depends to some extent on the concentration of the acid in the aqueous layer. At low acid concentrations a pure hydrocarbon surface

referred to as showing the effect on surface tension of the sodium salts of the saturated fatty acids of carbon chains of varying lengths. The slopes of those curves and the effect of the compounds on surface tension become clear when the theories of molecular orientation and polar groups are understood.

Harkins likens a polar group to a metallic weight attached to logs of wood. If the metallic weight is kept constant and exceeds in specific gravity the weight of the log, the log will be immersed in water. This is what happens when methyl alcohol is dissolved in water. The affinity of the —OH group for water is so great as to overcome the effect of the hydrocarbon chain. If, however, the log of wood is increased in size or in length, we eventually reach a point where the weight no longer will submerge it and the wooden end of the log will project above the surface of the water, the weight at the bottom tending to hold the log in an erect position.

If we imagine a bar of liquid which we divide into two parts by an imaginary plane, when the imaginary plane is lifted, the upper layer rises with it and two surfaces appear where there was no surface previously. In these two surfaces we will have a rearrangement of the molecules of the liquid from a random distribution, such as is characteristic of the interior of liquids, to one where the molecules are oriented in a more or less orderly fashion. Figure 70 is a diagrammatic example of such surfaces.

Similarly, if we dissolve butyric acid in benzene and pour this solution upon a water surface, the water will have a greater affinity for the carboxyl group of the butyric acid than for the hydrocarbon chain, whereas, on the other hand, benzene will have a greater affinity for the hydrocarbon chain than for the carboxyl group, and these two opposing forces will cause an orientation of the butyric acid at the benzene-water interface, similar to the orientation diagrammatically shown in Fig. 71.

Polar liquids are, in general, mutually soluble, and slightly polar liquids, such as hexane or octane, are soluble in other slightly polar liquids but are relatively insoluble in very polar liquids. It is the old theorem that "like attracts like." In general, the introduction of polar groups causes hydrocarbons to become soluble in water when the hydrocarbon chains are short. As the length of the hydrocarbon chain increases, there is a decrease in the free energy of a water interface up to a point where a hydrocarbon-water interface is formed, after which the free energy of the interface remains essentially constant. As Langmuir has shown, the surface energy of the paraffin hydrocarbons

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probably occurs above C_{12}. At high acid concentrations the point may be lowered below C_{12}. The point likewise depends on the temperature, for, as the temperature increases, the area occupied by a single molecule becomes greater and the film expands like a two-dimensional gas.

from hexane to molten paraffin is essentially the same (46–48 ergs per square centimeter), although the molecular weights differ very greatly.

From the above hypotheses, it is evident that the solubility of one

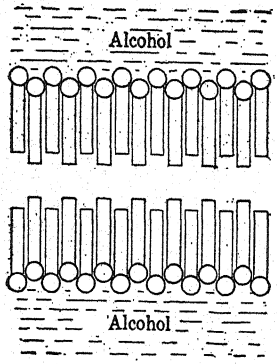


FIG. 70.

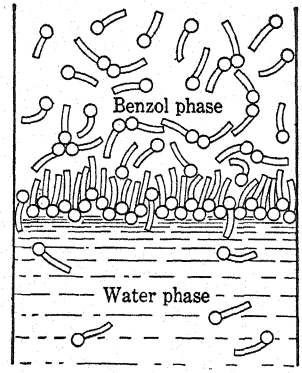


FIG. 71.

FIG. 70.—A diagrammatic representation of the orientation of molecules on surfaces when a column of alcohol is broken apart to give two surfaces. (After Harkins.)

FIG. 71.—A diagrammatic representation of butyric acid distributed between water, benzol, and the interface between the two. (After Harkins.)

substance in another may be the resultant of several opposing forces, or in other words, that *different parts of a molecule may possess different affinities toward the solvent.* If one studies the behavior of various compounds containing different groupings, it is possible to predict with a fairly high degree of accuracy the solubility behavior of an unknown compound.

Harkins, Clark, and Roberts measured the work which is required to pull apart a column of various organic liquids. This work is equal to twice the free surface energy of the liquid, inasmuch as two new surfaces are formed. They termed this the "cohesional work" for a bar of unit cross section. Values are also given for the "adhesional work" against water. The adhesional work is the work done when 1 sq. cm. of an organic liquid comes in contact with a similar area of water. This is a measure of the polar nature of the most polar part of the organic molecule. If $W_a/2$ is greater than $W_c/2$, then the work of attraction between the organic liquid and the water is greater than that between the parts of the organic liquid itself. Thus, the adhesional work is greater than the cohesional work. Consequently such substances should either dissolve more or less readily in water or should spread on a water surface. The actual magnitude of the values for the work of adhesion and the work of cohesion allows us to assign

numerical values to indicate the degree of polarity of the various radicals.

When oleic acid is placed on water, the —COOH groups are immersed in the water phase. The long hydrocarbon chains, however, have too much attraction for each other and too little for water to be drawn underneath the surface merely by the affinity of the —COOH group for water. Accordingly the oil spreads on the surface as a monomolecular layer. The spreading of an oil is thus due to the presence of a polar group in the molecule. Hardy found that many oils, such as the pure saturated aliphatic hydrocarbons, did not spread on a water surface, and explains this by stating that the great chemical stability of the paraffins makes chemical interaction with water impossible.

Harkins and McLaughlin [72] used values derived from a study of adhesional and cohesional energies and found 2.78×10^{14} molecules of butyric acid per sq. cm. of the interfacial film between hexane and water. This corresponds to a molecular area of 32Å^2.

Langmuir devised a so-called "surface-tension balance" which was later modified by Adam and others. Figure 72 shows one of the later

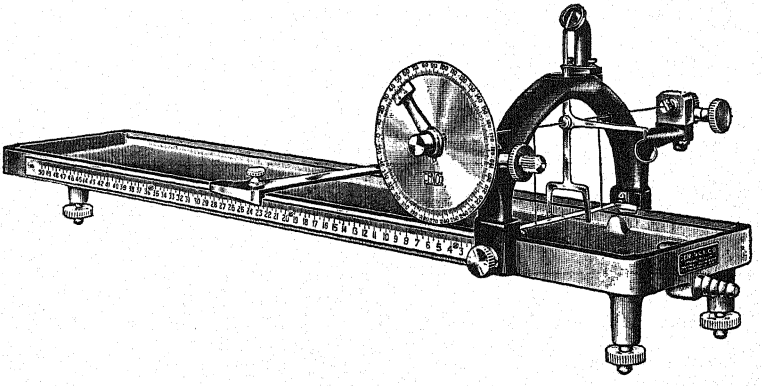


FIG. 72.—The hydrophile balance for measuring molecular areas and spreading characteristics of surface films.

models. This instrument is essentially a shallow trough with smooth, parallel sides. Suspended in the trough is a "float" which extends almost across the water surface and which is connected with the sides of the trough by extremely thin platinum or gold foil. This float acts as a movable barrier to prevent the escape of the film past it to the other end of the trough. The surface of the water in the trough is

[72] Harkins, W. D., and McLaughlin, H. M., Monomolecular Films between Liquids: Butyric Acid between Water and Hexane, and Acetic Acid between Water and Benzene, *J. Am. Chem. Soc.*, 47: 1610–1613 (1925).

swept clean of contaminants, and a small amount of surface-tension depressant is then placed upon the water surface. If this material spreads upon the surface (*i.e.*, the contact angle is essentially zero), it will form a monomolecular film. A movable barrier is now brought toward the film, compressing the film against the float. So long as the film is expanded, *i.e.*, the molecules in the film are not in contact with one another, the movable float will not change position. The movable barrier is advanced until a deflection of the float takes place. The instrument shown in Fig. 72 is designed to measure the pressure which the film exerts against the float, and the data so obtained can be converted into dynes per centimeter. Since the area of the film can be exactly measured and since we can also measure with exactness the weight of matter which was added to produce the film, we can calculate the number of molecules occupied by each square centimeter of the monomolecular film, and we can also measure, by continuing to move the movable barrier, the force which is necessary to cause the film to crumple and finally to collapse. The apparatus figured is also very useful for the determination of the spreading tendency of a liquid, since a compressed film will exert a definite pressure against the movable float and will continue to expand until the film is monomolecular.

Table XXX shows certain of the data for the surface area occupied

TABLE XXX

CROSS-SECTIONAL DIMENSIONS OF THE AREA OCCUPIED BY A SINGLE MOLECULE IN AN INTERFACIAL FILM (DATA OF ADAM)

Group	Cross Section, Å	Group	Cross Section, Å
Hydrocarbon chain.....	20.7	$-CH_2NH_2CONH_2$.....	25.5
$-CH_2CH_2COOH$.....	25.1	$-C_6H_4OH$.....	23.8
$-CH = CHCOOH$.....	28.7	$-C_6H_4NHCOCH_3$.....	28.2 or 25.8†
$-CH_2CH_2COOC_2H_5$*.....	22	Triglycerides.....	63
$-CH = CHCOOC_3H_5$....	28.7	Glycol dipalmitate.....	42
$-CH_2OH$.....	21.7	Cholesterol.....	39
$-CONH_2$.....	<21	Hydrolecithin.....	53
$-CN$.....	27.5		

* Ethyl, methyl, and allyl esters pack into the same area.
† According to temperature.

by a single molecule containing various groups as determined by Adam,[73] using an apparatus similar to that shown in Fig. 72. It will be noted that the first eleven substances in the table show some differ-

[73] Adam, N. K., The Structure of Surface Films on Water, *J. Phys. Chem.*, 29: 87–101 (1925).

ences in cross-sectional area. Nevertheless they are relatively alike. When we pass, however, to a triglyceride, it is apparent that the glycerol portion of the molecule is lying flat in the interface with the three hydrocarbon radicals of the fatty acids projecting into the surface, inasmuch as the cross section of the triglyceride is approximately three times that of the hydrocarbon chains. Similarly, in the dipalmitate we have two hydrocarbon chains projecting into the surface, with the glycol lying horizontally beneath.

Harkins and McLaughlin,[74] and Harkins and Gilbert [75] studied the structure of films on aqueous salt solutions. They find the surface of a salt solution to be covered with a monomolecular water film which ranges in thickness from 4 Ångström units on a 0.1 *M* NaCl solution to 2.3 Ångström units on a 5.0 *M* NaCl solution. As we have already noted (Table XXVI), the surface tension of inorganic salts is relatively high; consequently an inorganic salt solution can decrease its free energy by bringing pure water molecules into the surface. Apparently, however, from these studies, the water film on the surface is monomolecular in thickness, the difference in cross section being explained by the intensity of molecular "packing."

From the above studies Harkins calculates that a water molecule occupies a volume equal to a cube 3.09×10^{-8} cm. on an edge. He notes, however, that in these monomolecular films we are dealing not with a static film but with a film to which molecules are continually being added and from which molecules are continually escaping. He illustrates the kinetic energy of the surface by the following statement: "If a water surface at 20° is kept in a vacuum, 7,000,000 molecules jump out each second from the area occupied by a single molecule, so that at equilibrium an enormous number of molecules jump out from, and jump back into, this area. However, the time required for the orientation of a molecule of this type is so minute that at any instant the percentage of more or less oriented molecules is probably very high."

Du Noüy [76, 77] from a study of surface tension calculated the linear dimensions of the molecules of such materials as sodium oleate, egg albumin, serum albumin, etc., on the assumption that they form mono-

[74] Harkins, W. D., and McLaughlin, H. M., The Structure of Films of Water on Salt Solutions. I. Surface Tension and Adsorption for Aqueous Solutions of Sodium Chloride, *J. Am. Chem. Soc.*, 47: 2083–2089 (1925).

[75] Harkins, W. D., and Gilbert, E. C., The Structure of Films of Water on Salt Solutions. II. The Surface Tension of Calcium Chloride Solutions at 25°, *J. Am. Chem. Soc.*, 48: 604–607 (1926).

[76] du Noüy, P. L., Surface Equilibria of Biological and Organic Colloids, Chemical Catalog Company, New York (1926).

[77] du Noüy, P. L., Some New Aspects of the Surface Tension of Colloidal Solutions Which Have Led to the Determination of Molecular Dimensions, Colloid Symposium Monograph, Vol. III, pp. 25–47 (1925).

molecular layers. He noted that the materials studied could be divided into three groups. One group gave a single minimal value for the decrease of surface energy; another group gave two minimal values at different dilutions; a third group gave three minimal values. He explains this by assuming that in the first group the molecules had the form essentially of a cube, all three dimensions being approximately equal. Consequently, the area which would be covered by a unit weight of material in a monomolecular film would be identical regardless of the direction in which the molecule was oriented.

For the second group he assumed two dimensions to be essentially equal with an unequal third dimension. A given quantity of such material could form a monomolecular film by covering the surface with the molecules packed in such a way that their greatest dimension lay in the surface. However, on the addition of more material or by decreasing the surface area, the molecules would rearrange themselves to form a new monomolecular film with the square cross section in the surface and the greatest dimension of the molecule perpendicular to the surface.

The third group of substances he considered to have all three dimensions of the molecule unequal, thus allowing three monomolecular films to form, the first film with the molecules having their greatest dimension in the surface, on closer packing the intermediate dimension in the surface, and on closest packing the smallest dimension in the surface. The calculated dimensions for the molecules of sodium oleate were 6.64, 7.56, and 12.30 Ångström units, respectively. In crystalline egg albumin two of the dimensions were equal, the dimensions being 30.8, 30.8, and 41.7 Ångstrom units. Harkins,[78] from a study of the behavior of sodium oleate in an emulsion film, finds the area of the sodium oleate molecule to be 61Å.^{2}

Most physicochemical studies of molecular orientation have dealt with the formation of monomolecular films. Recently Blodgett,[79] working in Langmuir's laboratory, devised a technic for the production of polymolecular films and has built up polymolecular layers more than two hundred molecules deep. Blodgett uses a Langmuir surface tension balance upon which a surface-tension depressant (*e.g.*, the calcium or barium salts of palmitic, stearic, or other long-chain fatty acids) has been allowed to spread. This film is then compressed on the surface of the water by the spreading of another surface-tension depressant (*e.g.*, castor oil), the two films being kept separate by a waxed silk thread. In this way definite pressure can be brought to

[78] Harkins, W. D., and Beeman, N., *J. Am. Chem. Soc.*, 51: 1674–1694 (1929).

[79] Blodgett, Katharine B., Films Built by Depositing Successive Monomolecular Layers on a Solid Surface, *J. Am. Chem. Soc.*, 57: 1007–1022 (1935). (*Cf.* also Langmuir, I., and Schaefer, V. J., Composition of Fatty Acid Films on Water Containing Calcium or Barium Salts, *J. Am. Chem. Soc.*, 58: 284–287 (1936).)

bear on the calcium palmitate film by the spreading of the so-called "piston oil" (*e.g.*, the castor oil noted above). The pressure so produced (± 16.5 dynes per centimeter) is not sufficient to crumple the film but is sufficient to cause it to move upon another suitable surface upon which it can be spread. If now a clean glass or metal slide is *raised* through the film of calcium palmitate, the area occupied by the calcium palmitate on the water surface decreases by exactly the surface area of the slide, and a monomolecular layer of calcium palmitate is deposited over the surface of the slide with the carboxyl groups of the palmitic acid oriented toward the slide and the hydrocarbon "tails" extending outward from the slide. If now the slide is *lowered* through the calcium palmitate film, a second layer of calcium palmitate is deposited on the slide, in this instance the hydrocarbon portion of the molecule being attracted to the hydrocarbon surface already on the slide and the —COOH portion of the molecule extending in the surfaces. Thus films may be built up, one film at a time, by successive raising and lowering of the slide through the surface film, the 1–3–5–7 . . . films being hydrophobic and being wetted by oil and not wetted by water, and the 2–4–6–8 . . . films being hydrophilic and being wetted by water and not wetted by oil. Such polymolecular films when sufficiently thick show a beautiful series of interference colors. They may be dried even by baking in an oven without destruction of the film structure. Blodgett notes that Langmuir has suggested that "films could be built for use as diffraction gratings for soft X-rays by depositing $(2n + 1)$ layers of barium stearate, then $2n$ layers of stearic acid, then $2n$ layers of barium stearate, and so on in alternating succession. The stearic acid would be more transparent to radiations of short wave length than the barium stearate and would therefore serve to space the series of layers of barium stearate at known intervals apart."

Later studies [80] have dealt with built-up films of proteins and the properties of such protein films. By use of the Blodgett technic it has been found possible to build up multiple films of proteins of almost any thickness that may be desired. By use of the interference colors which such films show, and the number of layers of protein in the film being known, it is possible to measure with rather high precision the cross-sectional area of the protein molecule. Egg albumin gives a film about 20 Å in thickness, whereas a film of zein is only about 10 Å thick. When the film is deposited on a chromium-plated surface, egg albumin, pepsin, insulin, and the tobacco mosaic virus protein form mono layers, the "outside" of which is hydrophilic, *i.e.*, easily wetted by water but not easily wetted by oil, but in the case of casein and zein the mono layer is hydrophobic.

[80] Langmuir, I., Schaefer, V. J., and Wrinch, D. M., Built-up Films of Proteins and Their Properties, *Science*, 85: 76–80 (1937).

These studies, demonstrating beyond peradventure of a doubt the possibility of polymolecular films of oriented molecules, are regarded by the writer as of great importance in extending the theories of membrane formation, etc., in biological systems, and we shall have occasion to refer to Blodgett's studies again.

Another method of attacking the problem of molecular orientation is by studying the electrical behavior at interfaces. Compounds containing polar groupings are all electrical dipoles, and an oriented layer of dipoles should give rise to an electric double layer. Thus, in such systems we can study two problems at once, the electrical behavior of the double layer and the degree of specific orientation in the oriented molecular layer.

According to Gibbs' theorem (*vide infra*), surface-tension forces need not draw the molecule into the interface with any particular degree of orientation. However, electrical studies made on such surfaces

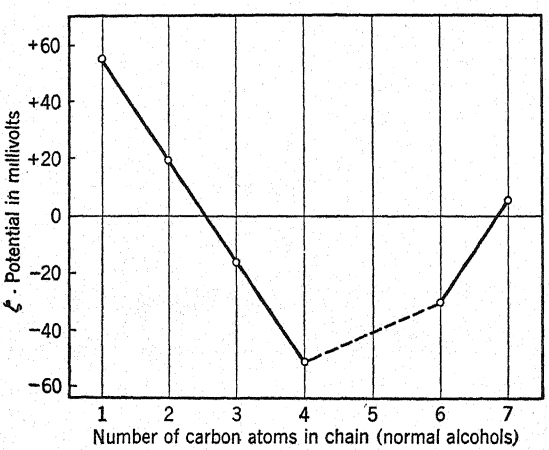


FIG. 73.—Showing relationship between the molecular structure of certain *normal*-aliphatic alcohols and the ζ-potential at a cellulose-alcohol interface.

may form a means of determining the extent to which the molecules in the surface layer have assumed any specific orientation. Accordingly the electrokinetic potential at the interface between cellulose or Al_2O_3 and a number of pure organic liquids has been studied [81-83] in

[81] Martin, W. McK., and Gortner, R. A., Studies on Electrokinetic Potentials. V. Interfacial Energy and the Molecular Structure of Organic Compounds. I. Electrokinetic Potentials at Cellulose-Organic Liquid Interfaces, *J. Phys. Chem.*, 34: 1509–1539 (1930).

[82] Jensen, O. G., and Gortner, R. A., Electrokinetics XII. Interfacial Energy and the Molecular Structure of Organic Compounds. II. Al_2O_3-Organic Liquid Interfaces, *J. Phys. Chem.*, 36: 3138–3151 (1932).

[83] Lauffer, M. A., Electric Forces at Organic Liquid-Solid Interfaces, Ph.D. thesis, University of Minnesota, June, 1937; *J. Phys. Chem.*, 42: (May 1938).

an attempt to relate the structure of organic compounds and the interfacial energy of such systems. It was found that the structure of the molecule apparently determined not only the sign but also the magnitude of the electrokinetic forces at the interface. Table XXXI shows

TABLE XXXI

ζ-POTENTIAL AND ELECTRIC MOMENT (δe) PER UNIT AREA OF THE DOUBLE LAYER AT INTERFACES BETWEEN CELLULOSE OR Al_2O_3 AND CERTAIN PURE ORGANIC LIQUIDS

	Cellulose-Liquid Interface		Al_2O_3-Liquid Interface		
	ζ-Potential mv.	(δe), e.s.u. × 10^6	ζ-Potential, mv.	(δe), e.s.u. × 10^6	Unbalanced Orientation of Molecules in Interface, per cent
Methyl alcohol	− 55.3	−464.1	− 26.7	−218.0	.....
Ethyl alcohol	− 19.9	−136.3	− 14.7	− 94.2	.....
n-Propyl alcohol	+ 17.1	+101.3	− 11.7	− 64.7	.....
Iso-Propyl alcohol	− 16.2	−112.8	+ 11.5	+ 73.6	.....
n-Butyl alcohol	+ 51.7	+265.2	− 3.7	− 17.8	.....
Iso-butyl alcohol	+ 12.4	+ 66.4	+ 2.0	+ 10.2	.....
n-Amyl alcohol	.....	.....	+ 8.9	+ 36.8	.....
n-Hexyl alcohol	+ 33.6	+118.4	+ 35.3	+122.4	.....
n-Heptyl alcohol	− 6.6	− 18.7	+ 8.9	+ 26.0	.....
Acetic acid	+ 5.27	+ 8.74	+ 39.4	+ 65.2	11.40
n-Propionic acid	+ 1.31	+ 1.12	+ 18.7	+ 16.0	2.34
n-Butyric acid	− 0.483	− 0.35	+ 17.4	+ 13.0	3.52
n-Valerianic acid	− 1.11	− 0.77	+ 12.1	+ 8.4	.....
n-Caproic acid	− 1.67	− 1.40	+ 29.0	+ 24.8	.....
Ethyl formate	− 6.31	− 11.80	+113.0	+212.0	24.10
Ethyl acetate	− 3.39	− 5.72	− 24.8	− 41.4	5.05
Ethyl *n*-propionate	− 1.96	− 3.02	− 19.1	− 29.0	3.56
Ethyl *n*-butyrate	− 2.04	− 2.85	− 32.1	− 43.6	.....
Benzene	0.00	.....	0.0	.....	.....
Methyl benzene	− 0.2	.....	.....	.....	.....
Chlor benzene	− 1.0	.....	.....	.....	.....
Brom benzene	− 6.7	.....	.....	.....	.....
Amino benzene	− 49.7	.....	.....	.....	.....
Nitro benzene	−142.0	.....	.....	.....	.....

certain of the data obtained. In the *n*-aliphatic alcohols a definite relationship was found to exist between the length of the hydrocarbon chain and the ζ-potential at the alcohol-cellulose interface. The introduction of a —CH_2-group into the main carbon chain changed the ζ-potential by approximately 35 mv. (*cf.* Fig. 73), whereas the intro-

duction of a $—CH_2—$ into a side chain (*e.g.*, replacement of a hydrogen by $—CH_3$) changed the ζ-potential by only approximately 4 mv.

In the case of cellulose the sign of the charge at the interface changes from minus to plus when we pass from ethyl alcohol to *n*-propyl alcohol. However, with an Al_2O_3-alcohol interface the change from minus to plus occurs between *n*-butyl and *n*-amyl alcohols. The *n*-aliphatic acids all gave positively charged interfaces with Al_2O_3, but the sign of the interface changes from positive to negative between *n*-propionic and *n*-butyric acids in the case of cellulose. Ethyl formate is the only ester which gives a positively charged interface and then only in the case of Al_2O_3, the corresponding cellulose interface being negatively charged.

The data on the aromatic compounds present many points of interest. Thus benzene, which possesses a completely symmetrical nonpolar configuration, showed no electrokinetic potential at the cellulose-liquid interface. Toluene, which is slightly unsymmetrical, showed a low but definite electrokinetic potential. Chlorobenzene, bromobenzene, aniline, and nitrobenzene showed increasing electrokinetic potentials, and it was suggested that studies such as this might be used to evaluate quantitatively the polarity of groupings substituted in a molecule.

In the last column of Table XXXI are certain calculations directed at ascertaining the degree of unbalanced orientation of the molecules in the interface. These calculations were based on the following assumptions. If organic dipoles are oriented at an interface, we might expect an arrangement more or less like that shown diagrammatically in Fig. 74. Assuming that the boundary between the movable and im-

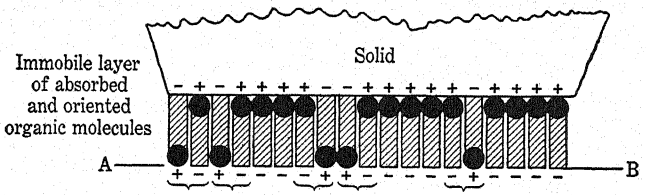


FIG. 74.—A diagrammatic representation of oriented dipoles at an interface postulated as the source of the ζ-potential.

movable liquid is at *AB*, the bracketed pairs of dipoles oriented in opposite directions might be expected to neutralize each other, whereas the "unbalanced orientation" of the remaining molecules should give rise to a net negative charge on the immovable layer side of the interface with a corresponding positive charge in the streaming liquid. On this hypothesis, it should be possible to calculate the percentage of "unbalanced orientation" of the organic molecules in the immovable layer, assuming (1) a monomolecular, close-packed oriented layer, and

(2) that the electric moment per unit area of the double layer is the product of the dipole moment of the organic molecule and the number of "unbalanced" molecules oriented per unit area.

In these calculations, the values for the cross-sectional area of the molecules (A) are those given by Rideal for the limiting areas per molecule in the liquid condensed form. For esters $A = 22.0$ Å^2, and for acids $A = 24.4$ Å^2.

The percentage of the total surface occupied by oriented but "unbalanced" molecules is given by the expression, $\delta e A/\mu \times 10^{-1}$,where μ is the molecular dipole moment, and δe and A have the meanings noted.

It will be seen that a relatively small percentage of unbalanced orientation is sufficient to account for the electric moment of the double layer.

If these studies are confirmed by further investigations and if the electrokinetic forces at a solid-organic liquid interface are due to the electrical dissymmetry of oriented organic molecules, the molecular orientation theory of Hardy, Harkins, Langmuir, Adam, *et al.*, can well be extended to include surface electrical forces as well as surface tension and interfacial tension.

It has been possible to indicate only in barest outline some of the theories and applications involved in the problem of molecular orientation. It is hoped, however, that sufficient has been given to stimulate the interest of the student and direct him to literature sources, for undoubtedly in this field of molecular orientation lies an explanation for many biochemical and biological phenomena.

ADSORPTION.—As noted above, a solution may decrease its surface energy by bringing into the surface either an excess of the solvent or an excess of the solute, depending upon which one causes a lowering of the surface tension. In the same way a colloid system can decrease its surface energy by bringing into the interfacial film substances which lower the interfacial tension. *Those substances which decrease surface energy tend to concentrate at a liquid-vapor interface, and those substances which decrease interfacial energy tend to concentrate at a liquid-solid or liquid-liquid interface. This phenomenon of concentrating at the interface is called* ADSORPTION.

Sodium oleate decreases the surface tension of water. If air is bubbled through a solution of sodium oleate, the foam which is carried over contains a higher percentage of sodium oleate than did the original sol, and the residual sol left behind is correspondingly more dilute. A dilute acetic acid solution or a solution of certain dyestuffs may be used equally well to demonstrate the same phenomenon.

The phenomenon of adsorption, as a rule, does not bring into play the forces of primary valence, *i.e.*, it is not possible to write a stoichiometrical chemical equation to represent the process, for, after all, *adsorption is an equilibrium* depending upon the concentration of the

material which is being adsorbed and the extent of surface upon which adsorption can take place.

Two general equations have been suggested as representing mathematically the characteristics of an adsorption reaction.

Freundlich [84] proposed an empirical equation from a fit of experimental data. His equation is:

$$\frac{x}{m} = aC^b \qquad (117)$$

where x = the weight of substance adsorbed, in grams;
m = the weight of adsorbent, in grams;
C = the concentration of the solution at equilibrium;
a and b = constants depending on the nature of the adsorbent and the substance which is adsorbed.

Equation (117) is the mathematical expression of a parabola and may be written:

$$\log \frac{x}{m} = \log a + b \log C \qquad (118)$$

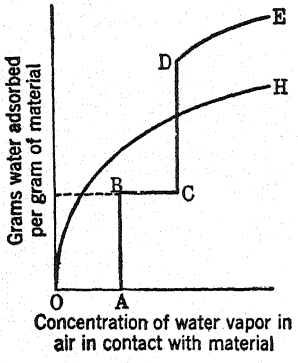


FIG. 75.—Contrasting the form of curves characteristic of surface phenomena with that characteristic of a chemical reaction. (After Bancroft.)

Surface phenomenon: Curve *O–H*—The adsorption of water vapor by dry charcoal.
Chemical reactions: Curve *ABCDE* — Behavior of Na_2SO_4 to water vapor.
OA = anhydrous Na_2SO_4.
AB = anhydrous Na_2SO_4 in contact with $Na_2SO_4 \cdot 10H_2O$.
Point *B* = only $Na_2SO_4 \cdot 10H_2O$.
CD = $Na_2SO_4 \cdot 10H_2O$ in contact with a saturated solution of Na_2SO_4.
Point *D* = a saturated solution of Na_2SO_4.
DE = varying concentrations (less than saturated) of Na_2SO_4.

The characteristic feature of the curve is that there is no single point where the reaction appears to be completed or where a new reaction begins. The system varies continuously and represents a reversible reaction.

Figure 75 is a diagrammatic representation of an adsorption curve, *OH*, as contrasted with a chemical reaction, curve *ABCDE*. In the curve *OH* we have a reaction such as occurs when water vapor is adsorbed upon the surface of dry charcoal or some other inert surface. In curve *ABCDE* we have a curve characteristic of the behavior of anhydrous sodium sulfate toward water vapor. Many chemical reactions appear to follow a parabolic curve. When, how-

[84] Freundlich, H., Kapillarchemie, Akademische Verlagsgesellschaft, Leipzig (1909).

ever, the data of such a reaction are plotted in terms of logarithms, the resulting curve is not a straight line. If the resulting curve is a straight line, we can be fairly sure that we are dealing with an adsorption reaction.

The constants a and b of the Freundlich adsorption isotherm can be determined by plotting, in terms of logarithms, the data of a reaction. The value of a is the value on the ordinate axis, where the straight line cuts the axis of ordinates at unit concentration, as shown in Fig. 76. The constant b is an expression denoting the slope of the curve and is the tangent of the angle θ which the line makes with a line drawn parallel to the axis of abscissa. It is an expression for the rate of adsorption,

$$\frac{d \log x/m}{d \log C}$$

when all variables except concentration have been held constant. Accordingly it is not necessary to plot the logarithmic curve in expressing graphically an adsorption reaction. Very extensive series of data can be concisely presented by tabulating the values for these two constants.

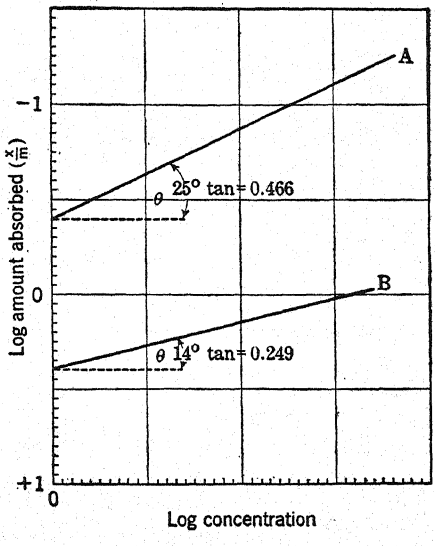


FIG. 76.—Showing the significance of the constants in the expression,

$$\frac{x}{m} = aC^b \quad \text{or} \quad \log \frac{x}{m} = \log a + b \log C.$$

Curve A,

$$\log \frac{x}{m} = -0.4 + 0.466 \log C.$$

Curve B,

$$\log \frac{x}{m} = 0.4 + 0.249 \log C.$$

The graphic method yields at the best only a close approximation for the value of these fundamental constants. Their absolute value may be determined by the method of least squares. Denoting values on the axis of ordinates as (y) and values on the axis of abscissa as (x),

$$a = \frac{\Sigma(x) \cdot \Sigma(xy) - \Sigma(x^2) \cdot \Sigma(y)}{[\Sigma(x)]^2 - n\Sigma(x^2)} \tag{119}$$

$$b = \frac{\Sigma(x) \cdot \Sigma(y) - n\Sigma(xy)}{[\Sigma(x)]^2 - n\Sigma(x^2)} \tag{120}$$

where Σ = the sum of the numbers involved;
n = the number of individual items which were summed.

Langmuir [85] proposed a different equation which may be stated as:

$$x = \frac{\alpha\beta C}{1 + \alpha C} \tag{121}$$

where x = the amount of material adsorbed;
C = the equilibrium concentration;
α and β = constants.

Equation (121) is the equation for a hyperbola and indicates that the interface will become saturated at some definite value of C. This value of C is determined by constant β which becomes equal to x when C is a maximum. This point is usually reached [86] when the equilibrium concentration is the concentration of a saturated solution.

In certain instances β may exceed the free solubility of a material. In discussing this point Brintzinger [87] presents the data shown in Table XXXII for the maximal adsorption of salicylic acid. He ex-

TABLE XXXII

ADSORPTION OF SALICYLIC ACID ON CHARCOAL AT VARIOUS TEMPERATURES

Temperature (° C.)	25	30	35	42	49	53
Solubility of acid (grams)	0.221	0.270	0.320	0.411	0.541	0.651
Acid adsorbed per gram charcoal	0.49	0.50	0.52	0.54	0.57	0.60

plains the excess adsorption over the true solubility as being due to a surface "pull" which is greater than the water "pull" on crystals of salicylic acid.

In the initial portions of the curves both the Freundlich and the Langmuir adsorption isotherms yield straight lines when plotted on logarithmic paper, so that over those portions of the curves there is little choice between the two mathematical expressions. Since, however, the Langmuir expression does indicate a definite adsorption saturation, it appears to be the preferable expression for adsorption studies.

The Mechanism of Adsorption.—There are in reality two schools of thought with respect to the phenomenon which we are discussing under

[85] Langmuir, I., The Adsorption of Gases on Plane Surfaces of Glass, Mica, and Platinum, *J. Am. Chem. Soc.*, 40: 1361–1403 (1918).

[86] Linner, E. R., and Gortner, R. A., Interfacial Energy and the Molecular Structure of Organic Compounds. III. The Effect of Organic Structure on Adsorbability, *J. Phys. Chem.*, 39: 35-67 (1935).

[87] Brintzinger, H., Schall, A., and Beier, H. G., Die maximale Adsorption I. Mitteilung: Die maximale Adsorption schwerlöslicher Säuren und Basen an verschiedene Aktivkohlen, *Kolloid Z.*, 74: 29–32 (1936).

the heading adsorption. To one of these schools, the ideas which will be propounded in this chapter, and even the term adsorption, are anathema. This school has considered primarily the behavior of electrolytes toward surfaces and is interested in such phenomena as acid-base exchange in soils and minerals, in acid and alkali binding of proteins, and in relationships which exist between the biocolloids and the electrolytes in biological systems and organisms. This school insists that stoichiometrical relationships account for the phenomena which we are calling adsorption and that the union between the electrolyte and the substrate is a true "salt" rather than an "adsorption complex."

Probably they are correct to a degree. It is well known that, in order to replace one equivalent of calcium or magnesium in a zeolite, it is necessary to add approximately two equivalents of an alkali metal. However, the various alkali metals differ among themselves in their replacement ability. Thus, Jenny [88] points out that there is a 240 per cent difference between the replacement ability of the lithium ion and the cesium ion on ammonium permutite and a 700 per cent difference in the replacement ability between the lithium ion and the potassium ion on hydrogen permutite, and that the monovalent ions arrange themselves in the lyotropic series $Li < Na < K < Rb < Cs < H$ with respect to permutite systems.

Apparently the binding takes place on *surfaces,* and the *extent of surface area* determines not necessarily the type of reaction that the system will undergo but rather the magnitude of the reaction which will take place. Unquestionably stoichiometrical chemical combination and surface adsorption in many instances involve the same chemical forces. This can be illustrated diagrammatically, if we take a hypothetical mass of carbon and project a plane through it. Assuming that the carbon atoms are arranged with a definite space relationship to each other, we might postulate an arrangement similar to that shown in Fig. 77. Those carbon atoms which are imbedded in the body of the carbon mass and are not exposed at any surface will obviously not take part in an adsorption or a stoichiometrical chemical reaction. Those carbon atoms which are exposed in the surface of the plane have one free valence bond and accordingly may enter into stoichiometrical reactions to a limited extent. Those carbon atoms which are exposed on edges are indicated as having two free valence bonds and accordingly may be expected to be more reactive than those atoms which are exposed only in surfaces. Those carbon atoms which are exposed at corners are indicated as having three free valence bonds, and accordingly such atoms should be more reactive than those which are exposed in edges and still more reactive than those exposed in surfaces. Thus, in order to prove the stoichiometrical nature of the bind-

[88] Jenny, Hans, Studies on the Mechanism of Ionic Exchange in Colloidal Aluminum Silicates, *J. Phys. Chem.*, 36: 2217–2258 (1932).

ing, it would be necessary to know not only the surface area of the solid phase but also the ratios which exist between the "atoms" which are involved in the binding and which are exposed at the "corners," at the "edges," and at the "faces" of the exposed planes, since those

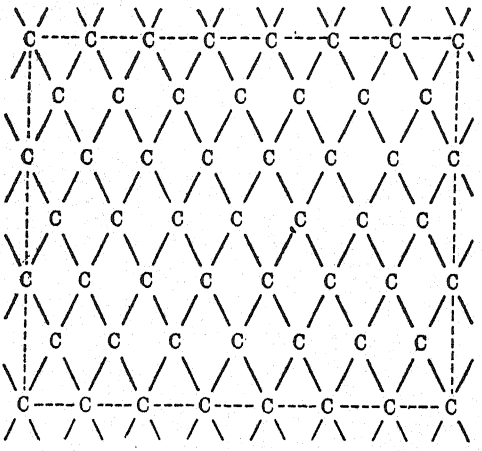


FIG. 77.—A diagrammatic representation of the unsatisfied valences at the sides and corners of a plane surface of space-oriented carbon atoms.

portions which constitute the "corners" will be theoretically more reactive than those which form the "edges," and these in turn will have a higher binding capacity than those in the "faces."

Taylor [89] has suggested that catalytic surface activity is due to surface atoms which have a high degree of valence freedom, thus approximating more nearly the gaseous state than the solid state, and that less than 1 per cent of the surface may be catalytically active. In addition, in surfaces we have those energies which have been designated as the van der Waals' forces. These forces differ only in degree from primary valence forces, since both are probably electrical in origin.[90]

Huggins [91] attempted to give a physical picture of the affinities causing adsorption and lists four energy sources: (*a*) an unpaired electron in the valence shell of an atom, (*b*) a positive atomic kernel (H, Na, Cu, etc.) not surrounded by electron pairs, (*c*) an electronegative

[89] Taylor, Hugh S., Fourth Report of the Committee on Contact Catalysis, *J. Phys. Chem.*, 30: 145–171 (1926), and Schwab, Georg-Maria, Catalysis, translated by H. S. Taylor and R. Spence, D. Van Nostrand Company, New York (1937).

[90] Herzfeld, K. F., and Smallwood, H. M., Chap. IV in Treatise on Physical Chemistry, edited by H. S. Taylor, Second Edition, D. Van Nostrand Company, New York (1931).

[91] Huggins, M. L., Adsorption Mechanisms, *Science*, 63: 454–455 (1926).

atom or a lone electron pair in the valence shell of a negative atom, and (*d*) double or triple bonds in which one of the bounding electron pairs is not near the line joining the centers of the two atoms which it holds together. Adsorption may result from (*ab*), (*ad*), and (*ac*) forces in carbon and fresh metal surfaces. Attractions (*bc*), (*bd*), (*dd*), and (*cd*) may take place in organic compounds. He notes that (*bd*) adsorption will be "polar," and that (*dd*) will probably be "apolar," and that adsorption in any specific case may involve all the above types.

Inasmuch as the rare gases are positively adsorbed by charcoal and show fairly high heats of adsorption (argon, 3,450 calories per mole at 0° C. as compared with 3,654 calories per mole for nitrogen and 1,870 calories per mole for hydrogen at the same temperature), it is evident that surface fixation and heats of adsorption do not necessarily reflect compound formation. The demonstrated adsorbability of the rare gases is respectfully referred to that group of workers who insist that all apparent adsorption reactions can be accounted for on the basis of stoichiometry.

Probably the truth actually lies somewhere in the intermediate ground between strictly stoichiometrical combination and purely physical surface adsorption. In the rare gases we are dealing wholly with surface behavior. In relatively inert organic molecules we are probably dealing largely with surface energies, and in electrolytes we have probably in part stoichiometrical unions with a residuum of surface energy attraction. Recently the proteins have been singled out as a great class of the biocolloids in which the assumption has been made that only stoichiometrical reactions occur. Thus, Loeb[92] and his school interpret the reactions of proteins with acids and bases wholly on the basis of stoichiometrical salt formation. However, there is evidence[93] that adsorption also accounts for at least a part of the apparent acid and base binding in protein systems, and it has been shown[94] that, in contrast to succinic acid, casein does not possess a "maximum base-binding capacity," but that together with the stoichiometrical binding of the base by the acid casein there is a second reaction which apparently yields an adsorption isotherm.

Recently, in order to account for the osmotic behavior of the red

[92] Loeb, J., Proteins and the Theory of Colloid Behavior, McGraw-Hill Book Company, New York (1922).

[93] Hoffman, W. F., and Gortner, R. A., Physico-Chemical Studies on Proteins. I. The Prolamines—Their Chemical Composition in Relation to Acid and Alkali Binding, Colloid Symposium Monograph, Vol. II, pp. 209–368, Chemical Catalog Company, New York (1925).

[94] Robinson, A. D., Gortner, R. A., and Palmer, L. S., Physico-Chemical Studies on Proteins VI. The Effect of Salts on the Casein-Sodium Hydroxide and Paracasein-Sodium Hydroxide Equilibria, *J. Phys. Chem.*, 36: 1857–1881 (1932).

blood cells, Peters [95] has suggested that the potassium "salt" of hemoglobin in the red blood corpuscles probably exists largely in an unionized form. It is difficult to picture a true potassium salt which fails to ionize. It is easy to picture an alternative potassium ion-hemoglobin adsorption complex which osmotically would behave as a unit, and that picture is suggested as an alternative to explain the osmotic behavior of the red cells.

Positive and Negative Adsorption.—Adsorption may be either positive or negative. *Positive adsorption from solution is the concentration of the solute in the interfacial film.* This concentration may reach high values and cause a very marked difference in the concentration of the original solution. *Negative adsorption is the concentration of the solvent in the surface film,* leaving an excess of the solute in the body of the liquid. Negative adsorption is always small in amount. Apparently in most cases only a monomolecular film of solvent is adsorbed at the interface. We have already noted that the pure water film on the surface of salt solutions is monomolecular.

The nature of the solvent is frequently of very great importance in determining not only whether or not adsorption will take place, but also the extent of the adsorption reaction. As a general rule, *adsorption is the greatest from solvents which have a high surface tension and least from solvents which have a low surface tension.* This is what one would expect, considering that surface energy interchanges are involved.

Willard Gibbs [96] predicted, long before experimental evidence was available, that substances which lower the surface tension or the interfacial tension would tend to concentrate in the surface film, because of the decrease in surface energy which would result. Gibbs' equation may be written:

$$C_2 = -\frac{C}{RT} \cdot \frac{d\gamma}{dC} \qquad (122)$$

where C_2 = the excess concentration in the interface;
C = the equilibrium concentration in the liquid phase;
dC = the increment of change in concentration of the solution for an increment of change ($d\gamma$) in the interfacial tension.

C_2 is positive when $d\gamma/dC$ is negative, *i.e.*, when the interfacial tension is decreased. Under such conditions we have positive adsorption. C_2 is negative when $d\gamma/dC$ is positive or when there is an increase in the interfacial tension of the system. Under such conditions

[95] Peters, J. B., Body Water—The Exchange of Fluids in Man, Charles C. Thomas, Springfield, Illinois, and Baltimore, Maryland (1935).

[96] The Scientific Papers of Willard Gibbs, Vol. I, Thermodynamics, Longmans, Green and Company, New York (1906).

we have negative adsorption. Bartell and Sloan (*loc. cit.*) restated Gibbs' theorem in terms of adhesion tensions as, *"There will be an excess in the concentration of the solute in the interfacial layer, if the ratio of change of the adhesion tension of the solution against the adsorbent is positive."*

Characteristics of the Adsorption Process.—It will be seen from equations (117) and (121) that the amount of material adsorbed is in direct proportion to the weight of the adsorbent, on the assumption that a portion of the same adsorbent in the same physical state is used. It would be more correct to say that *the amount of material adsorbed is directly proportional to the specific surface of a given adsorbent.* The measurement of specific surface is extremely difficult. In many instances it is practically impossible to determine the surface area of a given solid. However, provided that the substances are identical in all properties except extent of surface, the relative extent of surface can be determined through adsorption studies.

Garner, McKie, and Knight[97] used adsorption methods to determine the internal surface of charcoal and found a surface area of 66 sq. m. per gram. Paneth and Radu[98] found values for wood charcoal ranging from 46.2 to 268.0 sq. m. Harkins and Ewing[99] reached the conclusion that the internal area of 1 gram of charcoal is probably somewhat less than 120 sq. m. Incidentally they note that the intensity of adsorption of water on this charcoal surface is so great that the liquid is compressed to about 75 per cent of its original bulk, and they calculate that in order to bring about this compression a pressure in the neighborhood of 37,000 atmospheres would be necessary. This point will be referred to again.

Gustaver,[100] on the other hand, obtained much higher values of surface area. He considers the structure of charcoal as that of a mass of capillary spaces and that the adsorption of a vapor by charcoal consists of two stages: (*a*) the adsorption of the vapor at the interface, and (*b*) the condensed vapor then collecting in the form of liquid in the capillary pores. Because of this second stage, the equilibrium is not reached instantaneously. He concludes that 1 gram of charcoal possesses 600 sq. m. of surface area in the form of capillaries greater than 6×10^{-8} cm. in radius and that, when the finer capillaries are

[97] Garner, W. E., McKie, D., and Knight, B. C. J. G., The Area of the Internal Surface of Charcoal as Determined by the Adsorption of the Normal Aliphatic Alcohols from Aqueous Solution, *J. Phys. Chem.*, 31: 641–648 (1927).

[98] Paneth, F., and Radu, A., Über die Adsorption von Farbstoffen an Diamant, Kohle und Kunstseide, *Ber.*, 57: 1221–1225 (1924).

[99] Harkins, W. D., and Ewing, D. T., A High Pressure Due to Adsorption, and the Density and Volume Relations of Charcoal, *J. Am. Chem. Soç.*, 43: 1787–1802 (1921).

[100] Gustaver, B., Die Sorption von Dämpfen durch Kohle, *Kolloidchem. Beihefte*, 15: 185–338 (1922).

taken into consideration, the total surface area of 1 gram of charcoal may easily exceed 3000 sq. m.

Apparently the nature of the surface of a solid phase may determine in a large degree its behavior in acting as an adsorbent. We know that for charcoal to become an efficient adsorbent it must be "activated." The activation is carried out in several ways, one of the commoner ones being to heat the charcoal for a time in a current of steam and later heat it in a closed container to 700 to 800° C. No adequate proof has as yet been secured to explain the processes that take place in such "activation." However, it is improbable that such changes as occur involve the electrical double layer, since diamond dust, graphite, unactivated charcoal, and activated charcoal are all negatively charged and all possess about the same charge density.[101]

Effront[102] studied, among other systems, the adsorption of pepsin by pure cellulose (filter paper prepared for quantitative analysis). One would suppose *a priori* that filter paper made by different manufacturers would have identical adsorptive properties. Table XXXIII shows, however, that Effront found them to behave very differently toward pepsin.

TABLE XXXIII

ADSORPTION OF PEPSIN BY FILTER PAPERS OF VARIOUS MANUFACTURERS

Manufacturer	Units of Pepsin Adsorbed	Adsorption, per cent	Manufacturer	Units of Pepsin Adsorbed	Adsorption, per cent
Laurent.....	0	0	Dreverhoff, No. 417..	165	50
Berzelius.....	33	10	Dreverhoff, No. 402..	297	90
Schleicher, No. 589...	108	33	Dreverhoff, No. 311..	330	100
H. J. M. 100.....	128	39			

Such experiments indicate the errors which might be introduced into a study of the quantitative behavior of proteolytic enzymes when solutions which have been filtered through filter paper are used for enzyme study. Dreverhoff's paper No. 311 adsorbed all the pepsin from a given solution and allowed the accompanying contaminating proteins to pass through. On the other hand, the Laurent filter paper adsorbed selectively the proteins and allowed all of the pepsin to pass through.

[101] Frampton, V. L., and Gortner, R. A., Electrokinetics. XVIII. Interfacial Energy and the Molecular Structure of Organic Compounds. IV. The Electrokinetic Behavior of Charcoals in Aqueous Solutions of Organic Acids, *J. Phys. Chem.*, 41: 567–582 (1937).

[102] Effront, Jean, Contribution à l'étude du pouvoir adsorbant des tissus végétaux, *Ann. bull. sci. med. nat. Bruxelles*, 1926 (4): 24–178 (1926).

Neither the ash content nor any chemical analyses of the papers accounted for their different adsorptive behaviors.

The nature of the solvent is likewise of very great importance in adsorption reactions. As already noted, adsorption is greatest from solvents which have a high surface tension and least from solvents which have a low surface tension, due to the fact that a greater change ($d\gamma/dC$) can be brought about in a dispersions medium having high surface energy than in one having low surface energy. In many instances a substance will be positively adsorbed from water and be only slightly or not at all adsorbed from an organic solvent. Picric acid dissolved in water is very readily adsorbed on a charcoal surface, and can be removed completely from the aqueous phase by filtering off the charcoal. This charcoal-picric acid adsorption complex can be washed with large volumes of water without any appreciable quantity of picric acid appearing in the wash water. If, however, the charcoal-picric acid adsorption complex is washed with alcohol or with ether, the lower surface tension of the solvent causes the alcohol or the ether to replace the picric acid in the charcoal-water interfacial film, and the displaced picric acid moves out of the interface, giving a yellow solution in the alcohol or ether.

This reaction is of particular importance in the purification of organic compounds. Decolorizing a solution with boneblack or an activated vegetable carbon is a very common example of selective adsorption. After satisfactory decolorization has been secured, it often happens that the student washes, with an organic solvent, the carbon which has been filtered off, in order to be sure that he is not losing any appreciable amount of the valuable constituent which he wishes to conserve. In many instances these wash liquors are highly colored and contain the impurities which he originally wished to remove. Thus, the washing of the carbon adsorption complex with an organic solvent may entirely defeat the original purification program.

On the other hand, it may be possible to concentrate on an interface by the process of adsorption all the valuable constituent which is present in a solution, and then by later treating the adsorption complex with the proper solvent to remove the valuable constituent completely and obtain it in a highly concentrated and a relatively pure condition. Alkaloids can be quantitatively removed from an acid solution by "Lloyd's reagent" [103, 104] (hydrous aluminum silicate). The affinity of Lloyd's reagent for alkaloids is so great and adsorption is so complete that no bitter taste can be detected either in the mother liquor or in the Lloyd's reagent-alkaloid adsorption complex, when

[103] Lloyd, J. U., Process of Extracting, Purifying, or Excluding Alkaloids and Alkaloidal Salts, U. S. Patent 1,048,712. Dated Dec. 31, 1912.

[104] Lloyd, J. U., Discovery of the Alkaloidal Affinities of Hydrous Aluminum Silicate, *J. Am. Pharm. Assoc.*, 5: 381–390; 490–495 (1916).

such substances as quinine or strychnine are adsorbed. The adsorption complex can be filtered off, washed thoroughly with water so as to get rid of all contaminating sugars, salts, etc., and the alkaloid which was adsorbed upon the surface can be quantitatively liberated by dilute alkali, and can then be extracted from the solution by shaking out with chloroform, ether, etc. Similar examples of selective adsorption are numerous.

The adsorptive process is so general that care should be taken at every step in the preparation or the quantitative estimation of biochemical compounds in order to prevent adsorption reactions from interfering with the results which are desired. Accordingly *in quantitative studies it is never justifiable to decolorize a solution with carbon or with any other adsorbent unless preliminary experiments have definitely proved that such decolorization does not remove that constituent which is being quantitatively estimated.* This is especially important in experiments where the material is estimated by colorimetric methods. While it is essential that the original solution be colorless and clear, many of the methods which are used to produce clear, colorless solutions of biological fluids introduce errors due to forces of adsorption.[105]

The presence or absence of polar groups in the molecules of the solvent, of the solute or of the adsorbent may affect an adsorption reaction, probably by reason of their influence on the ease with which the surface of a solid adsorbent is wetted. Traube's rule, that surface-tension depression increases as we ascend an homologous series, has already been discussed in reference to Fig. 6. Freundlich[106] extended the rule to adsorption from aqueous solution and stated that *the adsorption of organic substances from aqueous solutions increases strongly and regularly as we ascend the homologous series.* Accordingly, in general, butyric acid is adsorbed more strongly than propionic, and propionic more strongly than acetic. However, *these generalizations are not invariably true.* Holmes and McKelvey[107] point out that Freundlich's adsorption studies dealt with carbon, a non-polar solid, in water, a polar liquid. They reversed the condition, using silica, a polar solid, and the various fatty acids dissolved in toluene, a non-polar liquid. They suggest that in Freundlich's studies the polar end (—COOH) of the fatty acid molecule was oriented toward the water phase and the hydrocarbon chain toward the carbon surface, whereas, in their own studies, the highly polar carboxyl end would be oriented

[105] *Cf.* Bock, J. C., A Study of a Decolorizing Carbon, *J. Am. Chem. Soc.*, 42: 1564–1569 (1920); Gortner, R. A., and Holm, G. E., The Colorimetric Estimation of Tyrosine by the Method of Folin and Denis, *J. Am. Chem. Soc.*, 42: 1678–1692 (1920).

[106] Freundlich, Herbert, Colloid and Capillary Chemistry, p. 195.

[107] Holmes, H. N., and McKelvey, J. B., The Reversal of Traube's Rule of Adsorption, *J. Phys. Chem.*, 32: 1522–1523 (1928).

toward the silica surface and the non-polar alkyl group toward the hydrocarbon solvent. They found a reversal of Traube's rule and that acetic acid was very strongly adsorbed from toluene on silica gel, propionic acid less strongly adsorbed, butyric acid still less adsorbed, and caprylic acid relatively slightly adsorbed. These observations afford another example of the role that molecular orientation plays at interfaces.

The adsorption process is an equilibrium i.e., if an adsorbing surface is brought into contact with a solution which has an equilibrium concentration of 0.01 N, and the adsorbing substance is then removed from that solution and placed in a more dilute solution which has an equilibrium concentration of 0.001 N, the amount of material adsorbed will be identical with the amount that would have been adsorbed at an equilibrium concentration 0.001 N. Or conversely, if an adsorbing material is removed from the solution where the equilibrium concentration is 0.001 N and placed in a stronger solution so that the equilibrium concentration is 0.01 N, the adsorbing material will take up exactly the same amount as would have been taken up had it originally been placed in the stronger solution. Accordingly one cannot filter off an adsorbent which has reached equilibrium in a chemical solution and wash it with a solution that has a concentration different from the equilibrium solution without changing the amount of material adsorbed on the adsorbing surface.

Adsorption reactions are, in general, characterized by a positive heat of adsorption. In some instances the amount of heat liberated is large and indicates the great affinity existing between the surface of the adsorbent and the material being adsorbed. Harkins and Ewing (*loc. cit.*) found that 1 gram of bone-charcoal gave a maximum heat of adsorption for water of 18.5 calories, and 1 gram of fuller's earth gave 32.0 calories. Lamb and Coolidge,[108] in an extensive study of the adsorption of various organic vapors on charcoal, found that the heat of adsorption could be expressed as

$$\log h = \log a + b \log x \qquad (123)$$

where h = total heat evolved during adsorption;
x = cubic centimeters of gas adsorbed per gram charcoal;
a and b = constants.

This expression is identical with the Freundlich equation (118) and indicates that heats of adsorption follow a true adsorption isotherm. When they plotted the logarithms of h and x, series of straight lines were obtained, all having the same slope (constant b) and differing only slightly in the position at which they intersect the ordinate axis

[108] Lamb, A. B., and Coolidge, A. S., The Heat of Absorption of Vapor on Charcoal, *J. Am. Chem. Soc.*, 42: 1146–1170 (1920).

(constant a). The fact that the vapors all gave parallel lines indicates that the same fundamental mechanism is operating in all cases. Over the range studied the heat of adsorption which was evolved for each successive increment decreased but slightly and was found to be expressed by the equation:

$$\frac{dh}{dx} = \frac{ab}{x^{(1-b)}} \tag{124}$$

It is evident that, when $(1 - b) = 0$, dh/dx becomes a constant, *i.e.*, the heat evolved becomes independent of the amount of vapor already adsorbed. For the systems studied $(1 - b)$ ranged from 0.044 to 0.100, indicating only a slight change in heat increment. The film was postulated to be polymolecular and for CS_2 was calculated to be approximately 40 molecules deep. The attractive force holding the molecules of the adsorbed vapor on the charcoal was calculated to be in the neighborhood of 37,000 atmospheres, when 1 cc. of liquid was adsorbed on 10 grams of charcoal. The heat of adsorption is therefore in reality the heat of compression.

Keyes and Marshall [109] studied the heat of adsorption of various gases on charcoal and found the following values: ether 7250, ammonia 6456, carbon dioxide 5450, methane 4600 calories per mole. They also note that in the adsorption of gases one is not limited to a monomolecular layer. They reason that the initial reaction forms a monomolecular layer of adsorbed gases upon the surface of the adsorbent, and that "the first and succeeding layers, because of their special state, constitute new adsorptive surfaces which may adsorb molecules of the same species as the first layer or molecules of a different species. Each succeeding layer (the adsorbing molecules all of the same species) partakes of the special state of the first layer in lessened degree until finally a layer is reached wherein the molecular state differs little from what may be imagined as a molecular 'contact' arrangement."

Nutting [110] calculated the differential curve (dc/dz), where c is the energy in calories evolved by the adsorption of water on a square centimeter $\times 10^4$ of a silica surface, and z equals the thickness of the adsorbed water film in centimeters $\times 10^{-6}$.

Figure 78 shows that the differential curve turns sharply at a thickness of water film between 4 and 5×10^{-6} cm., representing a layer of water 100–120 molecular diameters deep, and that the energy is not by any means confined to the first molecular layer, nor is there any apparent break after the first molecular layer has been formed. He

[109] Keyes, F. G., and Marshall, M. J., The Heats of Adsorption of Several Gases and Vapors on Charcoal, *J. Am. Chem. Soc.*, 49: 156–173 (1927).

[110] Nutting, P. G., The Adsorptive Force of Silica for Water, *J. Phys. Chem.*, 31: 531–534 (1927).

calculates the maximum pressure at the surface to be of the order of 17,410 atmospheres and adds that, if the solid pulls on the adsorbed liquid with a pressure equal to 17,000 atmospheres, the liquid must also pull on the solid to the same degree and accordingly *the pull of water on a quartz surface approximates the tensile strength of quartz!* This leads to a hydration and dispersion of quartz by water which is adsorbed onto the surface.

Bartell and Almy [111] measured the heat of wetting of activated silica gel and found that it was markedly decreased if the silica gel was dehydrated, falling from 18.55 calories per gram for a gel containing 3.18 per cent water to 9.97 calories per gram when the water content was reduced to 0.83 per cent.

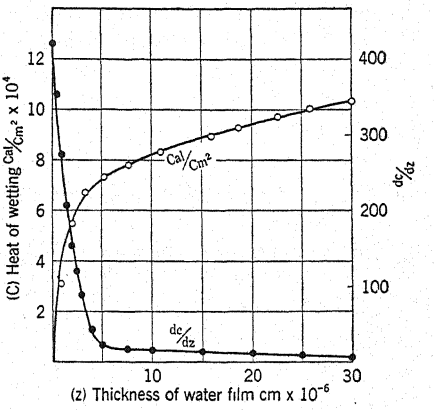


FIG. 78.—Thermal changes as related to the thickness of the water film on silica gel. (Data of Nutting.)

If heat is evolved by the adsorption of water upon a surface, at least an equivalent amount of heat must be added before the adsorbed water is released from the surface. From the heat of adsorption of liquids Patrick and Grimm [112] calculate a surface area of 6.9×10^6 sq. cm. per gram of silica gel. Neuhausen and Patrick [113] heated a silica gel to 300° C. *in vacuo* for a period of 6 hours without reducing the water content below 4.8 per cent, and Bartell and Almy state, "Water persists within the gel at temperatures well above the critical temperature of water." The adsorptive force of silica gel for water is so great that even at high temperatures water would be positively adsorbed over the interface. Such water, at least up to 4.36 per cent water in the gel, is under a high state of compression and has a density greater than liquid water.[114] Later under "bound water" we will discuss in detail the state of water as it exists in these adsorbed films.

Adsorption reactions are characterized by a negative temperature

[111] Bartell, F. E., and Almy, E. G., Activated Silica Gel. The Relation of Activity to Water Content and Temperature of Activation, *J. Phys. Chem.*, 36: 475–489 (1932).

[112] Patrick, W. A., and Grimm, F. V., Heat of Wetting of Silica Gel, *J. Am. Chem. Soc.*, 43: 2144–2150 (1921).

[113] Neuhausen, B. S., and Patrick, W. A., Organogels of Silicic Acid, *J. Am. Chem. Soc.*, 43: 1844–1846 (1921).

[114] Ewing, D. T., and Spurway, C. H., The Density of Water Adsorbed on Silica Gel, *J. Am. Chem. Soc.*, 52: 4635–4641 (1930).

coefficient. This is to be expected, provided that there is a positive heat of adsorption. The fact that adsorption becomes less as the temperature increases is probably due to the increased kinetic energy of molecules with an increase in temperature. An adsorption reaction can accordingly, in some instances at least, be distinguished from a chemical reaction, in that a decrease in rate of adsorption and a decrease in the amount of material adsorbed occurs at a higher temperature, whereas a classical chemical reaction is characterized by an increase in rate with an increase in temperature, although in many instances the same equilibrium is reached. Accordingly the constant of the Langmuir and Freundlich equations may be different at different temperatures.

PSEUDO-ADSORPTION.—True adsorption is an equilibrium, and diluting the system shifts the equilibrium concentration to the same point that would have been reached had the reaction been carried out originally at the lower concentration. It occasionally happens, however, that what appears to be an adsorption reaction is not a true equilibrium and is not reversible. For example, if porous porcelain is placed in a solution of auric chloride, gold is adsorbed from the solution which cannot be subsequently dissolved out of the adsorbing surfaces. The reaction in this case is probably similar to the reaction which Latshaw and Reyerson[115] utilize to prepare metallized silica gels. When silica gel is placed in solutions of salts of certain metals, including the noble metals, the salts are decomposed and the surface of the silica gel becomes covered with a thin layer of metal. Apparently the initial reaction is the adsorption of the metallic salt at the interface, and this adsorption is followed by a reduction of the salt by hydrogen (or other reducing substances) adsorbed on the surface of the silica gel. Accordingly the concentration of the metallic salt in the interface is altered, and additional adsorption takes place from the solution until finally by repetition of these processes the solution is almost entirely depleted of metallic ions. Graham, in 1830, noted that similar reductions occur on the surface of charcoal.

When an aqueous solution of egg albumin is beaten to a foam, the protein is positively adsorbed at the gas-liquid interface and *the surface energy coagulates the adsorbed egg albumin in the same way that egg albumin can be coagulated by heat.* Such denatured albumin will no longer dissolve in water, and accordingly we have an insoluble film of albumin which persists and prevents a reversal of the adsorption equilibrium. By stirring or bubbling air through a dilute solution of egg albumin, the protein can be removed practically quantitatively, and if the foam is allowed to stand until it breaks and is then filtered,

[115] Latshaw, M., and Reyerson, L. H., The Reducing Action of Hydrogen Adsorbed in Silica Gel, *J. Am. Chem. Soc.*, 47: 610–612 (1925).

it is possible to collect nearly all the original egg albumin in the form of insoluble films.

Pseudo-adsorption can, therefore, be defined as adsorption followed by a chemical change in the material which has been adsorbed, the chemical change being of such a nature as to inhibit the reversal of the adsorption process.

Pseudo-adsorption probably plays a role in the "poisoning" of hydrogen electrodes by Hg, Cu, As, etc. The ions are probably reduced by the hydrogen, so that we are no longer dealing with a platinum surface but rather with a mercury, copper, or arsenic surface which gives an erroneous or an unstable potential. Arsenic is notoriously poisonous to the platinum catalyst used in the contact process for the manufacture of sulfuric acid. Here again the explanation probably lies in the adsorption upon the platinum surface of arsenic or arsenic compounds, causing the surface to become catalytically inert. Biological catalysts may be "poisoned" by a similar process.

Catalysis by Adsorption.—We have already noted Taylor's view in regard to the nature of an active surface of nickel used in hydrogenation, *i.e.*, that we have certain free valences at isolated points in the catalytic surface, where atoms of nickel behave more or less as gaseous nickel. Almquist[116] takes a somewhat similar view of the nature of the surface of iron catalysts used in the synthesis of ammonia from nitrogen and hydrogen.

Boswell and Dilworth,[117] in a study of the role which aluminum oxide plays in the catalysis of certain organic reactions, such as the formation of ethylene and water from ethyl alcohol, point out that all the reactions which are catalyzed by aluminum oxide involve either hydrogen or hydroxyl ions, or the addition of water to, or the removal of water from, the reacting compounds, and reach the conclusion that aluminum oxide functions by means of a surface film of water which is the real seat of the catalysis. "There is still a water film on the surface, even after heating at atmospheric pressure at 500° C. for twenty hours, followed by two days' heating with a Meker burner." In regard to catalysis they conclude that *"The catalyst from this point of view does not accelerate a reaction already in progress . . . but actually initiates the change from one to the other."* Taylor has made a similar statement that a catalyst may initiate a reaction because of the energy residing in the catalytic surface, and when we discuss enzyme reactions, we will see that a combination (adsorption) between the enzyme and substrate usually is the first reaction to occur.

Some Applications of Adsorption.—The adsorption of gases on surfaces became of extreme importance when gas warfare was intro-

[116] Almquist, J. A., The Nature of the Catalyst Surface and the Effect of Promoters, *J. Am. Chem. Soc.*, 48: 2820–2826 (1926).

[117] Boswell, M. C., and Dilworth, H. M., On the Mechanism of Catalysis by Aluminium Oxide, *J. Phys. Chem.*, 29: 1489–1506 (1925).

duced. The problem of offense was to use poison gases which were not readily adsorbed upon the charcoal or other adsorbents used in the canisters of the gas masks, whereas the problem of defense was to prepare quickly suitable adsorbents for the gases which were introduced. In many instances, special adsorbents were found, and throughout the entire period of the World War, there was a rapid advance in the development of new adsorbents and improving the adsorbing power of the common materials, such as charcoal, silica gel, etc. Since the War the knowledge gained in this way has been applied to industrial problems in removing vapors of solvents, etc., from the atmosphere of factories, for the purpose either of reducing the health hazards or of recovering valuable solvents.

In the manufacture of sulfuric acid by the contact process, adsorption plays a very important role. The oxygen and sulfur dioxide are adsorbed upon the contact surface so strongly that they are brought within the radius of chemical attraction and unite to form sulfur trioxide which is less strongly adsorbed than the initial reacting materials. The surface accordingly is supersaturated with respect to the sulfur trioxide. Sulfur trioxide leaves the surface, allowing room for additional adsorption of oxygen and sulfur dioxide. Such a cycle will continue indefinitely as long as the surface remains in its original condition. The poisoning of such a surface is undoubtedly due to the irreversible adsorption of some foreign material on the catalyst where it is held with a greater tenacity than the products of the reaction which it is proposed to catalyze, that portion of the surface which is covered by the adsorbed poison film becoming catalytically inert.

Adsorption is used to de-ink magazines and old newspapers, making the paper available for re-use as newsprint. A small amount of bentonite mixed with pulped newspapers or magazines selectively adsorbs the printer's ink from the fiber, and when the mixture is run over wire screens and washed with water, the bentonite and the adsorbed printer's ink are readily washed from the fiber, yielding a reclaimed pulp which is practically ink-free and which can be made into a satisfactory sheet of paper with very little loss of the original fiber content.

The phenomenon of selective adsorption or rather of the wettability of surfaces by liquids is strikingly illustrated in the ore flotation process. Sulfide ores, such as copper sulfide and lead sulfide, have a non-polar surface and are not easily wetted by polar liquids like water. On the other hand, they are rather easily wetted by non-polar liquids, such as oils. Silica and silicious minerals, on the other hand, having a polar surface, are readily wetted by water but are not readily wetted by oils. If a rock, containing small amounts of valuable sulfides intermixed with large amounts of silicates, is finely ground and the ground mixture agitated with water to which a certain amount of oil has been added, the oil is selectively adsorbed by the sulfide particles,

so that they become coated with a film of oil, or a number of the sulfide particles adhere to a single oil droplet. If air is then blown through this mixture, the finely ground sulfides adhering to the oil particles rise to the surface in a foam which can be skimmed off, the silica and silicates of the gangue settling to the bottom of the agitator tank. In this way a relatively small amount of a valuable mineral may be separated from a relatively large amount of inert material. A concentrated high-grade ore is thus obtained. The ore flotation process has made it possible to utilize ores of much lower grade than was previously feasible. As a matter of fact, it has made possible the working of large masses of residues which had been earlier discarded. More than 60,000,000 tons of ore are concentrated in the United States each year by the flotation process.

De Groote[118] uses the theories of molecular orientation to break natural crude-oil emulsions on the theory that, if an emulsion of water-in-oil is stabilized by oleophilic compounds, such an emulsion may be broken by adding hydrophilic colloids. He has been very successful in this attempt by adding various types of organic acids (used as their ammonium salt) to crude-oil emulsions, tank bottoms, etc. Among the compounds which have been found most successful are diricinoleic acid [CH_3—$(CH_2)_5$—$CH(OH)$—CH_2—CH=CH—$(CH_2)_7$—CO—O—CH(—CH_2—$(CH_2)_4$—CH_3)—CH_2—CH=CH—$(CH_2)_7$—$COOH$], acetyl ricinoleic acid, monophthalyl ricinoleic acid, oxalyl ricinoleic acid, "oleic acid hydrogen hydrogen sulfate" [CH_3—$(CH_2)_7$—CH_2—$CH(O$—$SO_3H)$—$(CH_2)_7$—$COOH$], oleosulfonic acid [CH_3—$(CH_2)_7$—$CH(SO_3H)$—$CH(OH)$—$(CH_2)_7$—$COOH$], benzenesulfostearic acid [CH_3—$(CH_2)_7$—$CH(C_6H_4SO_3H)$—CH_2—$(CH_2)_7$—$COOH$], and dibutylnaphthalenesulfonic acid. It will be noted that these compounds differ only slightly in the internal balance of their polar and non-polar groupings. Nevertheless De Groote reports that a particular crude-oil emulsion may be readily broken by one of these compounds and be relatively unaffected by the remainder, and that each crude-oil emulsion presents its own individual problems and no blanket prescription will apply to all.

An important application of adsorption and apparently molecular orientation is afforded by the protection which small amounts of amines confer upon an iron surface in inhibiting corrosion.[119] Pieces

[118] De Groote, Melvin, Chemical Demulsification of Crude Petroleum, pp. 616–629, in "The Science of Petroleum," Vol. I, Oxford University Press, England (1937).

[119] Mann, C. A., Lauer, B. E., and Hultin, C. T., Organic Inhibitors of Corrosion. I. Aliphatic Amines, *Ind. Eng. Chem.*, 28: 159–163 (1936); II. Aromatic Amines, *Ind. Eng. Chem.*, 28: 1048–1051 (1936).

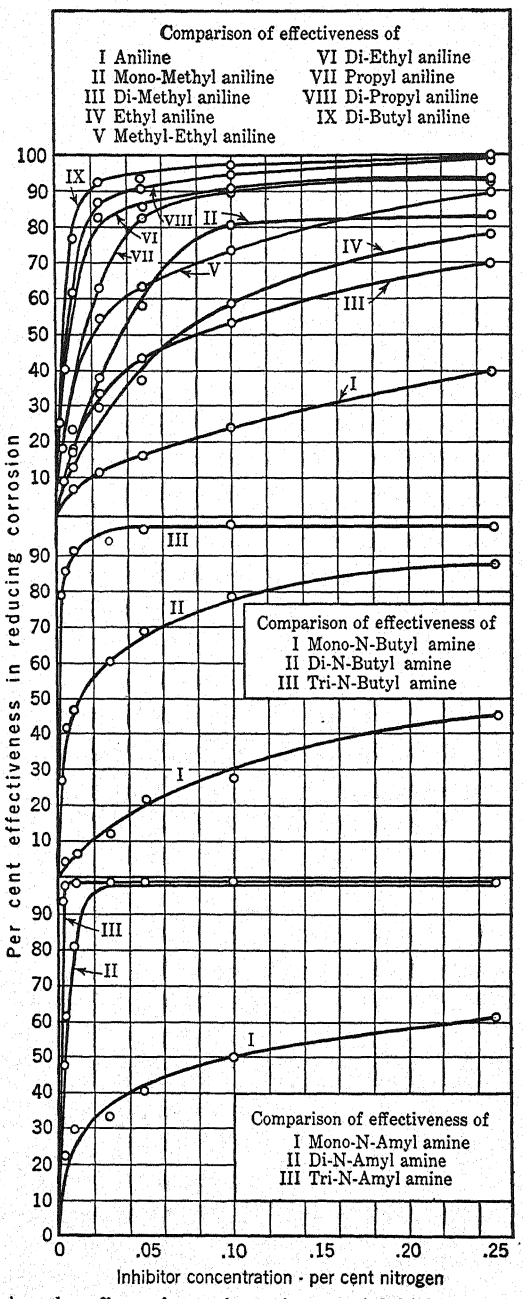


FIG. 79.—Showing the effect of certain amines on inhibiting corrosion of iron in an acid solution. (Data of Mann *et al.*)

of steel were immersed in *N*-sulfuric acid containing various amines and the rate of corrosion measured by weighing the steel sheet from time to time. Ammonium sulfate was relatively ineffective, and the inhibition of corrosion increased progressively from methylamine through ethyl, *n*-propyl, *n*-butyl to *n*-amylamine which was the most effective of the primary amines. Secondary amines were more effective than primary amines, and, with the exception of trimethylamine, tertiary amines were more effective than secondary amines. In some instances corrosion was inhibited 99.9 per cent in solutions containing as little as 0.005 per cent amine nitrogen. Somewhat similar results were obtained in the case of the aromatic amines, di-*n*-butyl aniline being particularly effective. Figure 79 shows the behavior of a few of the amines studied and illustrates the relationship between chemical constitution and surface behavior.

THE ADSORPTION OF WATER ON BIOLOGICAL PRODUCTS.—The hydrophilic colloids, characteristic of biological systems, possess a great affinity for water. The major phenomena involved will be discussed later when we consider the problem of gels. At this point it will be sufficient to consider only the problems pertaining to the drying of biological materials.

The determination of the moisture content of a biological material is a purely empirical procedure determined by the three variables, temperature, pressure, and time. In order to make a definite statement that such and such a biological material has such and such a moisture content, it is necessary to define the conditions in regard to temperature, pressure, and time of drying under which the moisture determination was carried out. *The removal of water from a biocolloid is merely shifting one equilibrium between a colloid surface and water to a new equilibrium*, and the extent to which the equilibrium is shifted is determined by these three variables. This has been emphasized by Nelson and Hulett,[120] who in a study of this problem conclude that moisture is retained by biological products at least to temperatures as high as 365° C., the critical temperature of water. Their studies will be referred to again under "bound water."

ENZYMATIC ACTIVITY.—Apparently the first reaction between an enzyme and its substrate is an adsorption of the enzyme by the substrate. This is followed by the chemical action of the enzyme. The initial adsorption reaction can be readily demonstrated. If pepsin is added to a suspension of fibrin in 0.2 per cent hydrochloric acid, and the mixture is vigorously shaken and immediately filtered, it will be found that the pepsin has been quantitatively adsorbed by the fibrin. This can be proved by the addition of fresh fibrin to the acid filtrate, under which conditions no appreciable digestion of the fibrin will be

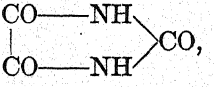
[120] Nelson, O. A., and Hulett, G. A., The Moisture Content of Cereals, *J. Ind. Eng. Chem.*, 12: 40–45 (1920).

observed. If fresh acid is added to the fibrin granules which were filtered off, hydrolysis proceeds, and the fibrin will become completely digested. The initial combination of the enzyme with the substrate usually takes place almost instantaneously, the rapidity of reaction and other characteristics indicating rather conclusively that it is a purely adsorption phenomenon.

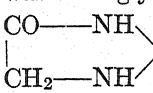
Quastel [121] emphasizes adsorption as an essential feature of enzyme action and accounts for specificity on this basis. He studied the oxidation⇄reduction reactions of bacteria on 108 organic compounds. Of these, 56 were "activated" so that they acted as hydrogen donators, *i.e.*, the hydrogen atoms in the molecule were transferable to a suitable "acceptor" such as oxygen, methylene blue, etc. Strict specificity of enzyme action would require 56 dehydrogenases. Quastel found that the 56 compounds could be grouped into classes, *e.g.*, a formic acid class, a lactic acid class, a succinic acid class, etc. He shows that *the rate of oxidation⇄reduction of any compound in a given class is altered (lessened) by the presence in the system of any other compound of the same class grouping but is unchanged in the presence of a compound belonging to another class.*

Quastel assumes adsorption at "active centers" on the bacterial membrane surface, these "centers" being *specific* for certain *molecular groupings.* Thus the "lactic acid center" adsorbs the grouping —CO—COH*— or —CHOH—COH*—, where H* is mobile. Glycollic, oxalic, glyoxylic, hydroxymalonic, glyceric, α-hydroxybutyric, mandelic, and pyruvic acids are all "adsorbed" and "activated" at the "lactic acid center," and in a mixture of two or more of these compounds there is competition for the area available for adsorption.

The "succinic acid center" is apparently specific for the grouping —C—CH_2COOH or —C—CH—COOH and in addition to succinic acid is the locus for adsorption of malonic, glutaric, phenylpropionic, tricarballylic, and pyrotartaric acids, all of which compete with one another for adsorption and for "activation" but none of which interfere noticeably with the "lactic acid group" of compounds. The "formic acid center" appears to be specific for formic acid. Even acetic acid does not interfere with reactions occurring at this center. An excellent demonstration of the specificity of adsorption as determined by chemical configuration is afforded by the fact that malonic acid is adsorbed and activated at the succinic acid center, hydroxy malonic acid at the lactic acid center, but ethyl malonic acid is not adsorbed at any of the centers. Furthermore, parabanic acid,



[121] Quastel, J. H., The Mechanism of Bacterial Action, *Trans. Faraday Soc.*, 26: 853–864 (1930).

was strongly adsorbed at the lactic acid center, whereas hydantoin,  , was not adsorbed, the hydrogenation of one carbonyl group destroying the specificity.

In summing up the work Quastel states, "Substances appear to act as 'poisons' simply by competing with the substrate for the space available for adsorption at the centers. The 'poison' and the substrate apparently compete with each other for adsorption on fairly equal terms. . . . A relatively large number of substances can be adsorbed in this specific manner, but out of this large number only a few can be *activated* to function as donators of hydrogen. . . . Specificity of enzyme action is seen to depend upon three factors:

"(1) Specificity of adsorption at the active centre.

"(2) The nature and strength of the polarizing field at the active centre.

"(3) The constitution of the adsorbed molecule.

" . . . The reason therefore why an enzyme is so specific in its action is, in the first place, because only a limited number of substances—containing a certain type of structure—is accessible to or adsorbed by the enzyme, and in the second place, because out of this limited number of substances specifically adsorbed only a few are capable of being turned into the 'active' molecules capable of the reactions under investigation."

Glick and King[122] have extended Quastel's studies in an investigation of the effect of the structure of organic compounds on the inhibition of the action of liver esterase. They found inhibition to increase rapidly as the length of the carbon chain of the *n*-aliphatic alcohols increased. One part of nonyl alcohol caused the same inhibition as 840 parts of methyl alcohol. When various groups were attached to the *n*-amyl alkyl radical, a lyotropic series of $CN > I > NO_3 > SH > Br > OH > Cl > CO > CONH_2 > NH_2$ was obtained from the inhibiting effect. One part of amyl cyanide was as effective as 270 parts of amylamine. When the alkyl radical was replaced by C_6H_5—, the inhibition followed a series which was only slightly altered, i.e., $I > Br > OH > Cl > NO_2 > CN > CH_3 > CONH_2 > NH_2$. One part of phenyl iodide was as effective as 58 parts of aniline.

Quastel's view of active centers is still further extended by the observation that coupled reactions occur in biological systems.[123] On

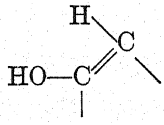
[122] Glick, D., and King, C. G., Relationships between the Structure of Saturated Aliphatic Alcohols and Their Inhibiting Effect upon Liver Esterase, *J. Biol. Chem.*, 94: 497–505 (1931); Relationships between the Structure of Organic Compounds and Their Inhibiting Effect upon Liver Esterase. Resemblance to a Lyotropic Series of Anions, *J. Biol. Chem.*, 95: 477–482 (1932).

[123] Schott, H. F., and Borsook, H., Coupled Reactions in Biological Systems, *Science*, 77: 589 (1933).

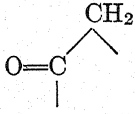
the surface of *Bacterium coli*, pyruvate is reduced to lactate by means of the energy of the anaerobic oxidation of formate to bicarbonate, and fumarate is reduced to succinate through the anaerobic oxidation of lactate to pyruvate. In both instances an intermediary energy carrier is necessary. This carrier must be reducible at the locus where one of the compounds is oxidized and reoxidized at the locus where the other compound is reduced. For the lactate-pyruvate-formate-bicarbonate system methylene violet serves as the intermediary, and methylene blue for the succinate-fumarate-lactate-pyruvate system. Without an intermediary no reaction occurs.

The bearing of the above experiments on various physiological and medical problems is obvious. We have as yet no specific information as to the types of surfaces which are exposed at the various centers of the bacterial mosaic surface to account for the areas characteristic of specific adsorption. It was in the hope of securing such specific information that the molecular orientation studies conducted in the author's laboratories were undertaken.

One other example from the field of medicine may suffice to illustrate the importance of specific adsorption and molecular orientation for biological studies. Morphine is well known to possess the desirable property of deadening pain but to have associated with this property the undesirable properties of inducing habit formation, producing nausea, and favoring constipation. The morphine molecule contains the grouping



Alvarez [124] notes that when the hydroxyl group is converted to a carbonyl group and the double bond is hydrogenated so as to yield a structure containing the grouping



the pain-deadening properties of the drug are increased about fivefold, the constipation and psychic effects are markedly decreased, and the habit-forming properties essentially disappear. In this case we are presumably dealing with adsorption and specific orientation of the original morphine on at least two brain centers, one which has to do with

[124] Alvarez, W. C., Dihydromorphinone Hydrochloride (Dilaudid, Bilhuber-Knoll): A Powerful Analgesic with Some Advantages Over Morphine, *Proc. Staff Mayo Clinic*, 7: 480–483 (1932).

habit formation and the other with pain. Apparently a slight change in chemical configuration of the molecule intensifies adsorption and orientation on the pain center and destroys adsorption affinities (or alters the specific orientation) of the drug on the habit-forming center. Admittedly the above explanation is a hypothesis, but in the light of Quastel's observations it appears to be an extremely logical explanation. We need specific adsorption and molecular orientation studies on all the physiologically active drugs and those compounds which are structurally closely related to them. It is believed that, when such data are available, the pharmacological and physiological behavior of drugs can be much more rationally interpreted.

CHAPTER VIII

ELECTROLYTES AND COLLOID SYSTEMS

We have already noted that a colloid micelle is stabilized by an electric double layer and that when the double layer is destroyed the micelle becomes isoelectric, and flocculation occurs. Solutions of electrolytes accordingly have a very marked effect upon the properties of colloidal systems. Those colloids which are *negatively charged* are rendered isoelectric and are flocculated by the *cations* of an added electrolyte, whereas *positively charged* micelles are sensitive to the added *anions*.

Referring to Fig. 5, the addition of Br^- causes the flocculation of positively charged silver bromide micelles until the isoelectric point is reached, following which a further addition of Br^- imparts a negative charge to the isoelectric silver bromide with the formation of negative silver bromide micelles. Accordingly, as an electrolyte is added to a colloidal sol, the electrokinetic potential is progressively decreased to zero. Further addition of electrolyte may cause the formation of a new sol possessing a charge opposite to that possessed by the original sol.

In general, the flocculating power of an ion follows the Hardy-Schulze rule that *"the precipitating power of an electrolyte depends upon the valence of the ion whose charge is opposite to that on the colloidal particle."*

Although the Hardy-Schulze rule is not a hard-and-fast one, it is in general true, and differences between ions of the same valence can probably be explained by differences in ionic radii, in degree of hydration of the ions, in ionic mobility, and in the degree to which they are adsorbed on the surface of the colloid micelle.

Negatively charged sols are coagulated by the cations, Na^+, Ca^{++}, Al^{+++}, and are but relatively slightly affected by the anions, Cl^-, $SO_4^=$, $PO_4^{\equiv}$, the reverse being true for the positively charged sols. *The influence of valence is not an arithmetical 1: 2: 3 ratio but more nearly a geometrical progression* $1 : x : x^2$, where, in some instances at least, x has a value lying somewhere between 16 and 32. If x should have a value of 32, it would mean that Ca^{++} would be 32 times as efficient a flocculating agent as Na^+ and that Al^{+++} would be 1,024 times as effective as Na^+.

The data in Table XXXIV explain the use of alum or iron sulfate in water purification. In large cities the water is frequently taken

TABLE XXXIV

EFFECT OF ELECTROLYTES IN FLOCCULATING FERRIC HYDROXIDE SOLS (DATA OF HARDY) AND ARSENOUS SULFIDE SOLS (DATA OF FREUNDLICH)

(Negative) Ferric Oxide Sols		(Positive) Arsenous Sulfide Sols	
Coagulating Ion and Salt Used	Coagulation Concentration, Gram Equivalents per Liter	Coagulating Ion and Salt Used	Coagulation Concentration, Millimoles per Liter
Cl^- (NaCl)	0.5	Na^+ (NaCl)	51.0
Cl^- (HCl)	0.5	Mg^{++} ($MgCl_2$)	0.717
SO_4^- (H_2SO_4)	0.002	Mg^{++} ($MgSO_4$)	0.810
SO_4^- (K_2SO_4)	0.0006	Ca^{++} ($CaCl_2$)	0.649
.....	.....	Ba^{++} ($BaCl_2$)	0.691
.....	.....	Al^{+++} ($AlCl_3$)	0.093

from a muddy river, pumped through a station where chlorine is added to destroy microorganisms and where iron or aluminum sulfate is added to flocculate the suspended particles. The water then goes on to the filter beds and passes through the filters into the water mains. Relatively small amounts of Al^{+++} or Fe^{+++} are required to clarify such waters. The clarification could be conducted by the addition of sodium chloride, but the amount of Na^+ that would have to be added to produce the same degree of flocculation would be so great that the water passing into the city main would probably taste salty, owing to the excess of sodium chloride remaining in the water.

Deltas are formed where rivers carrying clay and silt meet the salt water of the ocean, the clay and silt being deposited because of the neutralization of their electric double layer by the electrolytes in the ocean water.

The flocculating effect of electrolytes acting upon lyophobic colloids is due almost entirely to their effect on the stabilizing electrokinetic potential. We have already indicated, Fig. 60, that the electrokinetic potential does not have to drop to zero before the sol becomes unstable, but that instead there is a *critical zone* in the vicinity of the isoelectric point where the magnitude of the potential is not sufficiently great to insure indefinite stability.

Bungenberg de Jong,[1, 2] working in Kruyt's laboratory, has shown

[1] Bungenberg de Jong, H. G., Contribution to the Theory of Vegetable Tanning I. Dehydration of Lyophylic Sols and Gels by Tannins and Its Bearing on the Theory of Vegetable Tanning, *Rec. trav. chim.*, 42: 437–472 (1923).

[2] Bungenberg de Jong, H. G., Contributions to the Theory of Vegetable Tanning II. Dehydration of the Gelatine Sol by Tannic Acid, Crystalline Tannins and Simpler Phenols, *Rec. trav. chim.*, 43: 35–67 (1924).

that lyophilic sols are stabilized not only by an electric potential but likewise by solvation. Figure 80 represents his argument schematically. In *A* we have a neutral particle. This can become negatively charged, as in *B*, by the adsorption of an anion. The addition of a cation will reduce the potential to zero, and the particle will revert to its former condition as at *C*. The adsorption of a cation will produce a positively charged micelle at *D*, which again can be made isoelectric by the neutralizing effect of an electrolyte producing a particle, as at *E;* or the particle *A* can become solvated (or hydrated if the dispersions medium is water) as at *J*, and this solvated particle can, under the same conditions as above, become negatively charged, be neutralized, become positively charged, and again be neutralized without losing its liquid of solvation.

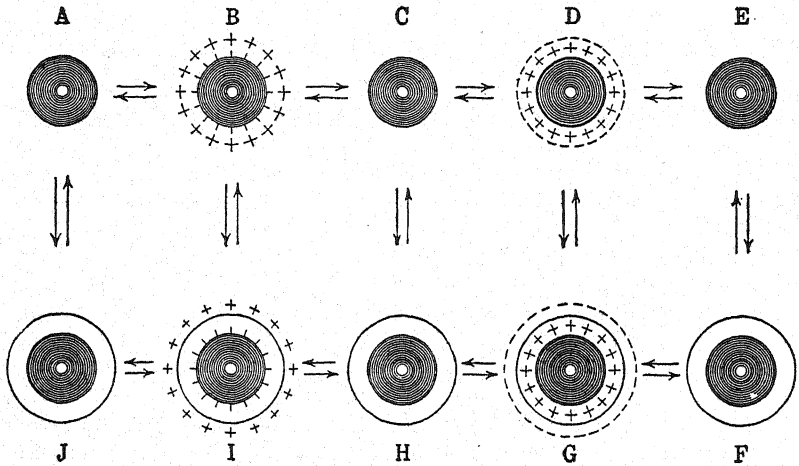


FIG. 80.—A diagrammatic representation of the relationships which exist between the solvation of a particle and the electrical charge of a particle, showing that both are important as factors influencing stability. *A–B–C–D–E* are lyophobic particles of which only *B* and *D* will exist in stable sols. *F–G–H–I–J* are lyophilic particles. All will be relatively stable as contrasted to the lyophobic series, but *G* and *I* have two factors for stability, solvation, and electric charge. Suitable electrolytes in low concentration will neutralize or even reverse the charges. "Desolvating" agents (*e.g.*, alcohol for water systems) will convert the solvated particles to lyophobic systems. (After Bungenberg de Jong.)

The addition of small amounts of electrolytes influences only the eelectrokinetic potential on the micelle. The addition of larger quantities of electrolytes influences the degree of solvation and causes flocculation to take place. In Fig. 80 flocculation will occur at *A, C,* and *E*. *B* and *D* will form stable lyophobic sols, *J, H,* and *F* isoelectric lyophilic sols possessing a considerable degree of stability, and *I* and *G* charged lyophilic sols having a high degree of stability. Dehydrating

agents, such as alcohol, will cause the lyophilic colloid to lose its liquid of solvation and accordingly effect the transformation from *I* to *B* or from *G* to *D*. Because of the influence which the liquid of solvation has upon the stability of a lyophilic system, the apparent effect of electrolytes on such systems is much less marked than on lyophobic sols.

The "salting out" of lyophilic colloids, such as proteins, from solution, by the addition of ammonium sulfate or by saturating the solution with magnesium sulfate is not an electrokinetic phenomenon but rather a dehydration of the neutral micelle whose electrokinetic potential has already been reduced to nearly zero by the first addition of the electrolyte but whose affinity for the solvent is overcome only in solutions which contain a large amount of the electrolyte where the ions of the electrolyte compete with the lyophilic colloid for the water which is available.

THIXOTROPY.—The term thixotropy was introduced in 1927 to describe those colloidal systems in which a gel is converted into a sol isothermally by shaking or stirring the gel, the sol setting to a gel again when left undisturbed. The term is derived from the Greek *thixis* (touching or striking), and *trepo* (to turn or to change). Almost all the literature dates since 1927, although prior to that time there were a few isolated observations of this peculiar behavior. Freundlich [3] and his students have been especially active in investigating this phenomenon.

There is a close relationship between thixotropy and coagulation. *Those ions which cause coagulation of a dilute sol bring about solidification and form thixotropic gels in more concentrated sols of those substances which are capable of showing thixotropic behavior.* For example, concentrated hydrous ferric oxide sols or concentrated hydrous aluminum oxide sols are converted into thixotropic gels by the proper concentration of anions, whereas concentrated, negatively charged sols of such materials as bentonite (a colloidal clay) are influenced chiefly by cations. Time of solidification of the sol for a given concentration of electrolyte is used to measure thixotropic behavior.

Thixotropic behavior depends upon a marked affinity existing between the particles and the liquid. The particles are surrounded by rather thick layers of liquid so that there is an interlocking between the particles when the system is at rest, and when the thixotropic gel is in equilibrium, the competition between the repelling forces and attracting forces results in the particles being rendered motionless. The result is a thixotropic gel. When the orientation of the particles with respect to each other is disturbed by shaking, the system loses its rigid-

[3] Freundlich, H., Thixotropy, Actualités scientifiques et industrielles, 267, Hermann et Cie., Éditeurs, Paris (1935), 50 pp., 83 references.

ity and acquires the properties of a sol. Since there must be a balance between attracting forces and repelling forces, the thixotropic behavior is realized only when the electrokinetic potential has been rather exactly adjusted to a rather narrow range.

Starch is a biocolloid which shows distinct thixotropic behavior. If a mass of starch is placed in a beaker and sufficient water added to cover the starch mass and the preparation allowed to stand for a few minutes, it will be found that considerable energy will be necessary to move a stirring rod through the mass. However, once stirring has been started, the mass becomes decidedly liquefied, and much less energy is necessary to continue the stirring. On standing again the starch-water system solidifies. The phenomenon of thixotropy is of major importance in the drilling of deep oil wells. A "drilling fluid" is usually injected into the well during the drilling operation to carry rock chips, etc., to the surface. If the drilling operation were to be suspended for a short period of time, these rock chips would settle to the bottom and form a compact mass around the drill bit so that it might be impossible to move the drill bit when operations were resumed. Accordingly, colloidal materials, such as bentonite, are added to the drilling fluid and so long as the bit is in operation the agitation keeps the drilling fluid in the sol form. If, however, drilling is suspended, the drilling fluid solidifies to a gel, and the rock fragments remain suspended in the gel. Drilling fluids have been devised where the sol-to-gel transformation occurs within less than 2 seconds after drilling operations have ceased. Quicksand in contrast to ordinary sand is thixotropic. It appears solid but when agitated becomes fluid.[4]

It is highly probable that protoplasm has thixotropic properties which may explain the ready sol $\rightleftharpoons$ gel transformations which so many biological workers have observed to take place in it. Chambers[5] comments on the fact that, when protoplasm is stirred with the fine needles of a micromanipulator, it becomes highly mobile but that it again becomes resistant to the movement of the needle if left undisturbed for a short period of time.

A few thixotropic systems exhibit the very surprising effect of solidifying rapidly if the sol is given a slow circular to-and-fro motion. This phenomenon has been called *rheopexy*.[6] Freundlich notes that in such systems the particles are rod- or plate-like and the slow circular motion appears to cause an orientation of the particles, and

[4] Freundlich, H., and Juliusburger, F., Quicksand as a Thixotropic System, *Trans. Faraday Soc.*, 31: 769–774 (1935).

[5] Chambers, R., The Effect of Experimentally Induced Changes in Consistency on Protoplasmic Movement, *Proc. Soc. Exp. Biol. Med.*, 19: 87–88 (1921).

[6] Freundlich, H., and Juliusburger, F., Thixotropy, Influenced by the Orientation of Anisometric Particles in Sols and Suspensions, *Trans. Faraday Soc.*, 31: 920–921 (1935).

that when the particles lie parallel to one another solidification takes place. Such sols solidify on standing but require a much longer time before solidification takes place than when they are gently rotated. Strong agitation again converts the system to a sol.

PROTECTIVE COLLOIDS.—Inasmuch as lyophilic colloids are stabilized not only by an electric double layer but also by the adsorbed dispersions medium, small amounts of lyophilic colloids are, in many instances, sufficient to "protect" lyophobic sols from the flocculating action of electrolytes. This behavior is known as *protective colloid action.*

Schulz and Zsigmondy [7] have given us a measure of the protective efficiency of lyophilic colloids in the *"gold number."* They define the gold number of a colloid as *that weight of the colloid in milligrams which will just fail to prevent a change in color from red to violet when 1 cc. of 10 per cent sodium chloride solution is added to 10 cc. of a Zsigmondy (formaldehyde) red gold sol to which the colloid has been added.* The mechanism of the reaction is an adsorption of the protective colloid on the surface of the gold micelles, so that the interface is no longer a gold-water interface but rather a hydrophilic colloid-water interface, the micelle acquiring the characteristics of the lyophilic colloid which was added. Table XXXV shows the gold numbers of various lyophilic colloids.

TABLE XXXV

GOLD NUMBERS OF VARIOUS LYOPHILIC COLLOIDS

Substance	Gold Number	Substance	Gold Number
Dextrin (British gum)...	125–150	Gum arabic.....	0.10 –0.125
Soluble starch.....	10–15	Protalbinic acid.....	0.15 –0.20
Sodium oleate.....	2–4	Lysalbinic acid.....	0.10 –0.125
Egg albumin.....	0.08–0.10	Gelatin.....	0.005–0.0125

Colloidal silver and colloidal silver oxide have pronounced bactericidal properties, and the "argyrol" of the physician is colloidal silver protected by protein split-products.

The protective action is characterized by a definite time interval. Sufficient time must elapse after the protective colloid has been added to the red gold sol to complete the initial adsorption of the protective colloid at the gold-water interface. Otherwise erroneous values will be obtained. The time interval before the sodium chloride is added, as a rule, need not exceed 10 minutes. If the protective colloid is

[7] Schulz, F. N., and Zsigmondy, R., Die Goldzahl und ihre Verwertbarkeit zur Charakterisierung von Eiweissstoffen, *Beitr. chem. physiol. Path.*, **3**: 137–160 (1903).

added to the solution of the electrolyte and then this mixture added to the colloid system, very much smaller quantities of electrolyte are sufficient to coagulate the lyophobic sol than would have been required if the protective colloid had first been added to the sol. The explanation probably lies in the fact that the colloidal micelles adsorb the protective colloid and form a micelle which is not readily flocculated. No such pronounced adsorption takes place between the solution of the electrolyte and the lyophilic colloid, and when this mixture is added to the lyophobic system, sufficient time does not elapse for the stabilizing adsorption to take place, flocculation occurring at once.

Various other measures of protective value have been suggested. Windisch and Bermann [8] proposed an *iron number* to characterize hydrophilic colloids which prevent the coagulation of Fe_2O_3 sols. They thus identified a gum in the wort as being responsible for stabilizing the foam of beer.

Wo. Ostwald [9] has suggested a *rubine number,* in which Congo rubine sols are used in place of the gold sol, the rubine number being defined as the amount of colloid in grams per 100 cc. of solution which prevents the change of color of a Congo rubine sol from red to blue. The electrolyte used is potassium chloride, 160 millimolar concentration.

Undoubtedly various hydrophilic colloids will give different values for the iron number, the gold number, and the rubine number, and within a group of hydrophilic colloids the various values for these numbers will not necessarily follow the same order. Only a few of the factors involved in protective action have been ascertained in a qualitative way, and as long as different lyophobic micelles possess different electrokinetic potentials and different interfacial tensions, they must possess different adsorptive capacities, and the molecules which are adsorbed are probably not always oriented in a similar manner. Because of these variations, it is probably too much to expect that gold numbers should exactly parallel the iron numbers, or that either one of these should parallel the rubine numbers.

If the electrokinetic potential on the hydrophobic micelle is opposite in sign to the potential on the hydrophilic colloid which is being adsorbed, protection is not conferred by small additions of the hydrophilic colloid, but rather the system is made very unstable, owing to a neutralization of the charge on the lyophobic micelle by the charge carried by the hydrophilic micelle.

The sequence of reactions is (1) increased sensitivity toward electrolytes, (2) neutralization of the potential, with resulting flocculation,

[8] Windisch, W., and Bermann, V., Über die Bedingungen für das Schaumen der Bierwürze, *Wochschr. Brau.*, 37: 129–132 (1920).

[9] Ostwald, Wo., Über "Schutzwirkungen" beim Elektrolyt-Umschlag des Kongorubins und über die "Rubinzahl" einiger organischer Kolloide, *Kolloidchem. Beihefte*, 10: 234–243 (1919).

and (3) if sufficient amount of the hydrophilic colloid is added, the reversal of the charge on the lyophobic micelle, and protection.

The gold number of cerebrospinal fluid was introduced into medicine a number of years ago as a diagnostic technic. Since the cerebrospinal fluid of patients suffering from general paresis of syphilitic origin shows a different flocculation area from that characterizing normal cerebrospinal fluid, Wright and Kermack [10] propose the use of gum benzoin sols for the diagnosis of cases of general paresis. Certain of their data are shown in Fig. 81. The area of precipitation shown by the vertical lines is the area characteristic of normal cerebrospinal fluid. The area denoted by the horizontal lines appears to be characteristic of cerebrospinal fluid from cases of general paresis. The constituents which cause flocculation of gum benzoin in this area are abnormal constituents not present in the cerebrospinal fluid of normal persons. Similar technics [11] have been proposed for the diagnosis of cancer.

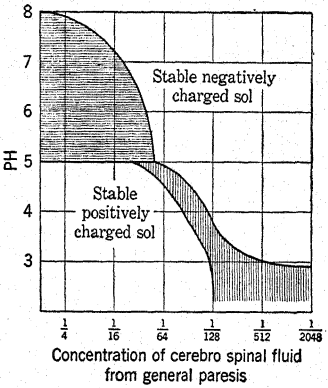


FIG. 81.—Showing the areas characteristic of flocculation of gum benzoin sols by cerebrospinal fluid from pathological cases. (Data of Wright and Kermack.)

MUTUAL PRECIPITATION OF COLLOIDS.—The preceding paragraphs have indicated that positively charged colloids will cause flocculation when added to systems containing negatively charged micelles, and *vice versa*. This property can often be utilized for the qualitative determination of the sign of the charge on the micelles in a given system. Filter paper (cellulose) is negatively charged against water, and if a strip of filter paper be dipped into a hydrosol, the liquid will be drawn up by capillary action. If the micelles present in the sol are negatively charged, they will ascend the strip of filter paper, although as a rule at a somewhat slower rate than the water. On the other hand, if the micelles are positively charged, their charge will be neutralized at the point where the filter paper touches the surface of the sol and only the dispersions medium will ascend by capillary action. The re-

[10] Wright, H. D., and Kermack, W. O., The Properties of Colloidal Gum Benzoin, *Biochem. J.*, 17: 635–657 (1923); The Mechanism of Precipitation of Colloidal Gum Benzoin by Cerebrospinal Fluid, *Biochem. J.*, 17: 658–667 (1923).

[11] Van den Bovenkamp, G. J., and Kisjes, W. H., Uitvlokking van Serumeiwitten door Natrium-Chromaat als Hulpmiddel bij de Kankerdiagnose, *Nederl. Tigjdschr. Geneeskunde*, 77, III: 2319–2327 (1933); Nadere Gegevens der Natriumchromaatpropionzuurreactie als Hulpmiddel bij de Kankerdiagnose, *Nederl. Tigjdschr. Geneeskunde*, 78, II: 2762–2766 (1934).

verse behavior would take place if the absorbing column were a positively charged colloid.

This method of determining the sign of the charge on colloid micelles is known as *capillary analysis* and can sometimes be advantageously applied to determine whether or not a chemical system contains only one kind of colloidal micelles. Thus, if a purple dye solution is tested, it may be found that the filter paper is colored a uniform purple color, in which case the dyestuff is probably a single chemical compound, or the filter paper may have a zone of red (or blue) advancing ahead of the purple boundary, in which case the purple solution was prepared by mixing red and blue dyestuffs. The purity of a dye can, as a rule, be roughly ascertained by this method, owing to a difference in rate of diffusion of the different dyestuff molecules.

Mutual precipitation of colloids is of extreme importance in the industry. Dyeing of fabrics is largely dependent upon the adsorption of the dye by the fiber. Animal fibers, such as wool, are amphoteric, *i.e.*, the charge on the fiber is rather readily altered so as to be either positive or negative, depending on the hydrogen-ion concentration of the medium. Animal fibers, therefore, can be dyed with either positive or negative dyestuffs by simply shifting the hydrogen-ion concentration of the dye bath.

Cotton possesses a negative charge which is not readily reversed by changes in hydrogen-ion concentration. Substantive dyes color cotton directly. These dyes, derivatives of the benzidine series, possess basic groups, and the micelles carry a positive charge. The attraction between dyestuff and fiber is, therefore, the attraction between positively charged and negatively charged colloids. Mutual precipitation occurs on or in the interstices of the fiber, and the adsorbed dyestuff is firmly fixed on the oppositely charged fiber.

In order to dye cotton or other negatively charged fibers with acid dyestuffs, it is necessary to mordant the fiber. The mordants commonly used are aluminum or chromium salts. The fiber is put through a mordanting bath where Al^{+++} or Cr^{+++} is adsorbed by the negatively charged fiber in such amounts that the original negative charge on the fiber is reversed by the positively charged metallic ions, the mordanted fiber acquiring a strong positive charge which permits of the adsorption and mutual precipitation of the negatively charged dyestuff on the surface and in the interstices of the fiber.

Moore[12, 13] utilized the principle of the mutual precipitation of colloids in preparing arsenical insecticides which adhere over long

[12] Moore, W., Spreading and Adherence of Arsenical Sprays, Minn. Agr. Exp. Sta., Technical Bull. 2, 50 pp. (1921).

[13] Moore, W., Adherent Arsenical Preparations, *Ind. Eng. Chem.*, 17: 465–466 (1925).

periods to the foliage of plants. Plant tissues in general possess a negative charge. Furthermore, leaf surfaces are covered with an adsorbed water film. Diagram *A*, Fig. 82, shows diagrammatically a positively charged particle neutralized by being adsorbed on a negatively charged surface, and the position which the water film would assume. Diagram *B*, Fig. 82, shows the hypothetical position which would be taken by a negatively charged particle sprayed onto a negatively charged surface, with the relative position of the water film. It is easily seen that the particle in *B* would be readily washed off of the surface by a stream of water. All the commercially available arsenicals were found to be negatively charged. Positively charged arsenicals were easily prepared, utilizing the principles involved in the complex theory of colloids. Laboratory tests of adherence showed that, if leaves were sprayed with the negatively charged arsenical and, after drying, were placed under a spray of water, practically all the arsenical was removed within a few minutes. However, if leaves were sprayed with the positively charged preparations, and then, while still wet, were immediately placed under a spray, a very considerable amount of the arsenic remained on the leaves after several hours' washing. Hooker[14] finds that colloidal copper hydroxide, owing to its positive charge, is an efficient fungicide when sprayed in a concentration of 1 part of the colloid to 5000 parts of water.



FIG. 82.—A diagrammatic representation of the relationships which exist between a leaf surface with its adsorbed water film and a particle of (*A*) a positively charged or (*B*) a negatively charged insecticide.

Powdered okra has been used with success in the treatment of gastric ulcer.[15] Its effectiveness appears to be due to the phenomenon of mutual precipitation. At the *p*H of gastric digestion (±*p*H 3) the gastric mucosa is positively charged. Okra contains a plant mucilage which is negatively charged at that *p*H. Apparently therefore the negatively charged carbohydrate slime is precipitated on the surface of the gastric mucosa, covers the ulcer, and prevents further abrasion by the gastric contents, thus relieving pain and affording conditions for rapid healing. Apparently the okra treatment is of no avail in cases of duodenal ulcer, which is in accord with the theory noted above, since at the *p*H of the duodenum the mucosa and the carbohydrate slime would both be negatively charged.

COACERVATION.—Although the phenomenon which is now known as

[14] Hooker, H. D., Colloidal Copper Hydroxide as a Fungicide, *Ind. Eng. Chem.*, 15: 1177–1178 (1923).

[15] Meyer, J., Seidmon, E. E., and Necheles, H., The Treatment of Peptic Ulcer with Powdered Okra, *Ill. Med. J.*, pp. 339–343 (Oct., 1933).

coacervation had been observed earlier, it remained for Kruyt[16] and his student, H. G. Bungenberg de Jong, first to describe the fundamental principles underlying the phenomenon. The word is derived from the Latin *acervus* (heap or swarm), combined with the prefix *co* (together), and means, literally, to swarm together. It has been allegorically compared to a swarm of bees which behaves as a unit but in which each bee retains its own individuality.

Coacervation is a phenomenon involving the lyophilic colloids and particularly the hydrophilic colloids. It has been extensively studied by Bungenberg de Jong[17] and his students. We have already noted that the hydrophilic colloids possess two factors of stability, an electric charge and a hydration shell. Many lyophilic sols may be brought to the isoelectric point and still remain in a relatively stable sol, stabilized by the hydration shell. The phenomenon of coacervation is the separation of microscopic liquid droplets when sols of two hydrophilic colloids of opposite electric charge are mixed. After a time these droplets may unite to form a viscous liquid layer at the bottom of the container. They constitute a new phase. Coacervation is a special application of the phenomenon of mutual precipitation. Gelatin and gum acacia sols may be taken as representative systems which form coacervates.[18] Figure 83 shows diagrammatically the conditions which must be met. Gelatin, in common with most proteins, assumes either a positive or a negative charge depending upon the hydrogen-ion concentration of the system. At a *p*H greater than 4.7 gelatin is negatively charged.

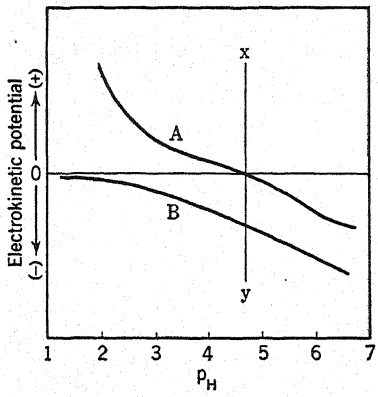


FIG. 83.—A diagrammatic representation of the relationship between *p*H and electro-kinetic potential for protein (*A*) and non-protein (*B*) sols. Coacervates will form at greater acidities than *p*H 4.7.

[16] Bungenberg de Jong, H. G., and Kruyt, H. R., Coacervation (Partial Miscibility in Colloid Systems), *Proc. Acad. Sci. Amsterdam*, 32: 849–856 (1929).

[17] See numerous papers in *Kolloid Zeitschrift, Kolloid Beihefte*, and *Biochemische Zeitschrift* since 1929. See also Bungenberg de Jong's summary, Bungenberg de Jong, H. G., La coacervation, les coacervats, et leur importance en biologie. I. Généralitiés et coacervats complexes; II. Coacervats auto-complexes, Actualités scientifiques et industrielles, Nos. 397 and 398, Hermann et Cie., Paris (1936), 54 pp. and 66 pp., and Koets, P., Coacervation of Amylophosphoric Acid and Proteins, *J. Phys. Chem.*, 40: 1191–1200 (1936).

[18] Holleman, L. W. J., Bungenberg de Jong, H. G., and Modderman, R. S. T., Über Koazervation I: Einfache Koazervation von Gelatinesolen, *Kolloid Beihefte*, 39: 334–420 (1934).

At *p*H's below 4.7 the micelles are positively charged. The reversal of sign of gelatin is shown by curve *A*, Fig. 83. Gum acacia retains its negative charge over a wide range of hydrogen-ion concentration, as indicated by curve *B*, Fig. 83. At the right of the line *xy* both gelatin and gum acacia are negatively charged. The two sys-

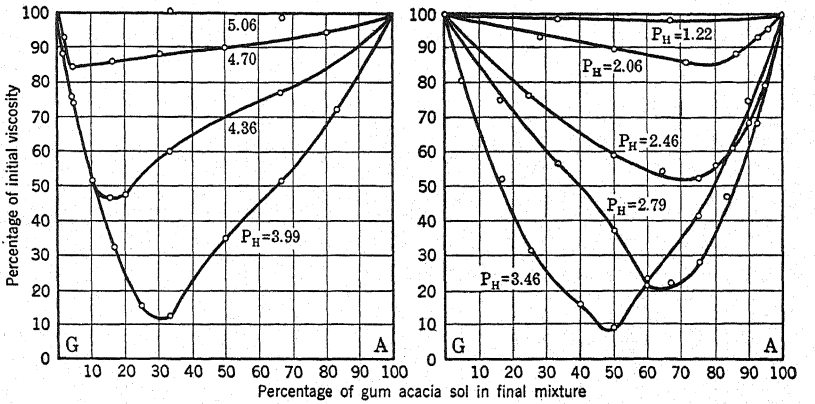


FIG. 84.—Showing the percentage viscosity-change of various mixtures of an 0.67 per cent gelatin sol (*G*) and an 0.67 per cent gum acacia sol (*A*) at various *p*H values, with particular reference to coacervate formation. (Data of Bungenberg de Jong.)

tems do not interact when mixed, and the relative viscosity of the mixture is the average of the relative viscosities of the sols used (note Fig. 84, system at *p*H 5.06). To the left of line *xy*, Fig. 83, mutual precipitation will occur between the gelatin and gum acacia micelles, but because of the water shell surrounding the individual micelles they cannot coalesce with complete destruction of the double layers but are held apart by the resistance of the water shells. *Electrostatic forces tend to cause the aggregation, but these forces are resisted by the elasticity of the water shells surrounding the hydrophilic micelles (cf. Fig. 85), so that the individual charged micelles retain their identity but are held together in a swarm by electrostatic attractions.* The net result is that droplets of liquid separate as a new phase.

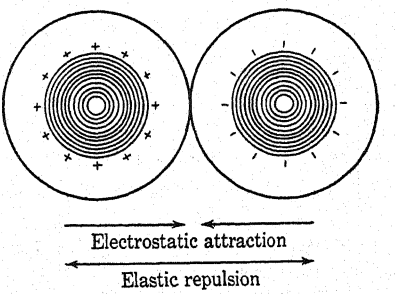


FIG. 85.—A diagrammatic representation of electrostatic attraction and elastic repulsion (of water shells) as the factors responsible for the phenomenon of coacervation.

Figure 86 [19] shows a mixture of the coacervate of positively charged gelatin and negatively charged gum acacia together with that of positively charged gelatin and negatively charged nucleic acid. Methyl green colors gelatin-nucleic acid coacervates but does not color gelatin-gum acacia coacervates. Accordingly mixtures of the two complexes can be distinguished as in Fig. 86. While for any given ratio of the sols, essentially isoelectric coacervate droplets may be obtained by the proper adjustment of the hydrogen-ion concentration, the individual micelles of which these droplets are composed retain their characteristic electric charge, so that a shift in the hydrogen-ion concentration or the addition of electrolytes to the system may cause the coacervate to redisperse into a sol composed of a mixture of the micelles of the original components of the coacervate.

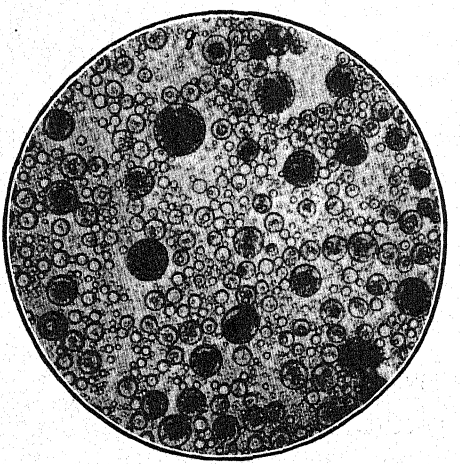


FIG. 86.—Showing mixed coacervate droplets formed by mixing gelatin (+), gum acacia (—), and nucleic acid (—) sols. The coacervate of gelatin-nucleic acid stains with methyl green; the coacervate of gelatin-gum acacia does not stain. (Data of Bungenberg de Jong.)

The real test as to whether or not a phase separating from a colloid-containing system is a coacervate depends upon the effects that salts may have upon this phase. Since the force of attraction which causes the coacervate to form depends upon the presence of electrokinetic potentials of opposite sign on the two reacting colloids, it is evident that the removal of the electrokinetic potential on either colloid destroys the attracting force and likewise the coacervate. On a colloid carrying a negative charge, the ζ-potential is most readily reduced by addition of polyvalent cations; a colloid carrying a positive charge is most susceptible to high valent anions. The sensitivity of the potential to salts in either case follows the Schulze-Hardy rule. The destruction of the ζ-potential on either component of a coacervate allows the separated phase to redisperse, *i.e.*, the force of mutual attraction disappears. *In a true coacervate the separated phase may be made to redisperse (at constant pH) by adding salts, and the effect will be found to follow a double*

[19] Bungenberg de Jong, H. G., and de Haan, A., Zur Kenntnis der Komplexkoazervation. XX. *Biochem. Z.*, 263: 33–49 (1933).

Schulze-Hardy rule, i.e., the effect is produced by *both* cations and anions.

In order for a coacervate to form, it is not necessary that the electrostatic forces of the two components be exactly balanced. Figure 87 illustrates this diagrammatically. As negatively charged gum acacia is added to a positively charged gelatin sol, the viscosity of the mixture progressively decreases until at point *B* the coacervate begins to separate. Between *B* and *C* the coacervate droplets retain a net positive charge, because the ζ-potential of the gelatin which they contain is greater than the ζ-potential of the gum acacia component. At *C* the electrokinetic potentials of the two components are exactly balanced, and the individual droplets are of themselves isoelectric, *although they still contain both positively charged and negatively charged micelles.* From *C* to *D* the potential on the gum acacia exceeds the potential of the gelatin, and the coacervate droplets as a whole possess an excess negative potential. In mixtures represented over the ranges *AB* and *DE* the electrokinetic charges and the kinetic energy of the individual micelles prevent the separation of a coacervate. The cross-hatched area of the diagram is the area within which a coacervate may be expected to separate.

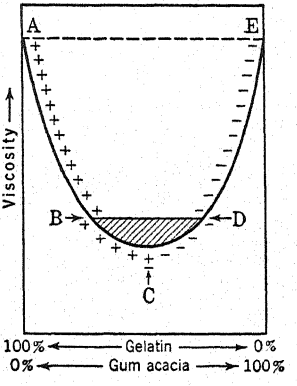


FIG. 87.—A diagrammatic representation of the effect of *p*H on the additive viscosity effects and coacervate formation when sols of gelatin and gum acacia are mixed. Dotted line (*AE*) = sols mixed at *p*H = 6.0, both sols (—) charged. Solid line = sols mixed at *p*H 3.5, gelatin (+), gum acacia (—). Coacervate droplets separate in area (*BCD*). Coacervate droplets are neutral electrically, *i.e.*, (+) $\cong$ (—), only at *C*.

The phenomenon of coacervation appears to be of enormous importance in biological systems. Bungenberg de Jong [20] considers that many of the phenomena of biology can be duplicated by model systems of coacervates. Protoplasm is an aqueous system containing hydrophilic colloids but retains its identity when immersed in water. Its behavior is remarkably paralleled by systems of coacervates. Complex coacervates on aging show the phenomenon of vacuole formation

[20] Bungenberg de Jong, H. G., Die Koazervation und ihre Bedeutung für die Biologie, *Protoplasma*, 15: 110–173 (1932); Models for the Stimulation of the Organ of Smell (with G. G. P. Saubert), *Proc. Acad. Sci. Amsterdam*, 40: 302–306 (1937); Phosphatide Auto-complex Coacervates as Ionic Systems and Their Relation to the Protoplasmic Membrane (with J. Bonner), *Proc. Acad. Sci. Amsterdam*, 38: 797–806 (1935); Phosphatide Auto-complex Coacervates as Ionic Systems and Their Relation to the Protoplasmic Membrane. II (with G. G. P. Saubert), *Proc. Acad. Sci. Amsterdam*, 40: 295–301 (1937).

and the extrusion of liquid from the coacervate droplet. Most of the lyophilic biocolloids readily form coacervates either with proteins or with themselves, since many of the biocolloids are zwitterions or contain groups some of which ionize as acids and others as bases (*e.g.*, phosphatides, proteins, nucleic acid, etc.). Accordingly, at the proper hydrogen-ion concentration or salt concentration, sols containing such compounds may concentrate into coacervate droplets. In some instances at least these coacervate droplets which separate have been confused with true liquid crystals. When we discuss the nucleoproteins we will see that they are systems of basic proteins combined with nucleic acid. Here we are possibly dealing with coacervate systems.

THE LYOTROPIC SERIES.—Valency is not the only factor involved in the behavior of ions toward a colloid system. A group of monovalent ions possesses within itself varying degrees of ability to flocculate sols. Hofmeister was the first to investigate this phenomenon. In a series of papers [21-23] from his laboratory he showed the effects of various anions and cations upon protein systems. Numerous workers have since made similar studies, using both lyophilic and lyophobic systems, and it is generally agreed that the anions can be arranged in a series of citrate > tartarate > SO_4 > acetate > Cl > NO_3 > Br > I > CNS, where, at least for protein systems, citrate shows the greatest precipitating effect, and thiocyanate the least. The cation effects are somewhat less marked but may be expressed approximately as Th > Al > H > Ba > Sr > Ca > K > Na > Li.

These or similar series of ionic effects have come to be known as the lyotropic series, or the Hofmeister, or the irregular series of ions. Various theories have been propounded to account for the observed differences in the behavior of the various salts. The behavior is not limited to colloid systems, since it has been abundantly demonstrated in other connections. Thus, Jaeger [24] finds the surface tension of molten alkali salts at 1000° C. to show a series of F > SO_4 > Cl > Br > NO_3 > I and Li > Na > K > Rb > Cs, and essentially the same order holds for the effect of these ions in increasing the surface tension of water. Freundlich suggested that in all probability the series correspond to the order of the hydration of the ions, the most hy-

[21] Hofmeister, F., Zur Lehre von der Wirkung der Salze. Zweite Mittheilung, *Arch. exptl. Path. Pharmakol.*, 24: 247–260 (1888).

[22] Lewith, S., Zur Lehre von der Wirkung der Salze, Erst Mittheilung, *Arch. exptl. Path. Pharmakol.*, 24: 1–16 (1888).

[23] Hofmeister, F., Zur Lehre von der Wirkung der Salze, Dritte Mittheilung, Fünfte Mittheilung, und Sechste Mittheilung, *Arch. exptl. Path. Pharmakol.*, 25: 1–30 (1889); 27: 395–413 (1890); 28: 210–238 (1891).

[24] Jaeger, F. M., Temperaturabhängigkeit der molekularen freien Oberfläschenenergie von Flüssigkeiten im Temperaturbereich von —80 bis +1650 C°., *Z. anorg. allgem. Chem.*, 101: 1–214 (1917).

drated ions being at the sulfate and lithium ends of the lyotropic series. Ionic radius likewise appears to be an important factor.

Frumkin [25] measured the potential difference at an air-solution interface. Table XXXVI shows certain of his data. The effect of the anion on the phase-boundary potential follows the order, F, SO_4 > Cl > Br > NO_3 > I > CNS, and he suggests that this series is related to the hydration of the anions. A similar view has been expressed by Kruyt and Robinson,[26] who suggest that molecular orientation may well be a factor. They note that the solubility of various materials

TABLE XXXVI

PHASE BOUNDARY POTENTIALS, ϵ, BETWEEN AIR AND THE SURFACE LAYER OF CERTAIN INORGANIC SALT SOLUTIONS (DATA OF FRUMKIN)

Electrolyte	Solution $N/1$, ϵ (millivolts)	Solution $2\,N$ ϵ (millivolts)	Electrolyte	Solution $N/1$, ϵ (millivolts)	Solution $2\,N$, ϵ (millivolts)
KF	.....	+ 5	Na_2CO_3	+ 3	+ 5
KCl	− 2	− 6	RbCl	.....	− 7
KBr	−10	−16	CsCl	.....	− 6
KI	−34	−52	LiCl	.....	− 9.5
KCNS	−57	−87	$Al_2(SO_4)_3$	0	+ 6
KOH	0	+ 5	HF	−71	−103
K_2CrO_4	.....	− 1	HCl	−23	− 55
K_2CO_3	.....	+ 3	HBr	−34	− 78
NaI	−39	−55	HI	−61	−112
$NaNO_3$	−17	−23	H_2SO_4	−13.5	− 55(?)
NaCl	− 1	− 4	H_3PO_4	− 0.5	
Na_2SO_4	+ 3				

may be very different in a salt solution from what it is in pure water. In the case of quinone they found a lyotropic series of anions influencing solubility, the solubility of quinone in a 1.5 molar solution of KCNS being 170 per cent of its solubility in water, whereas in the case of K_2SO_4 of an equivalent concentration the solubility was only 63.7 per cent of the water solubility. When hydroquinone was used, it was found that the cations showed the more pronounced lyotropic series, ranging from CsCl solutions, where the solubility of the hydroquinone was but slightly lower than in pure water, to LiCl, where the solubility

[25] Frumkin, A., Potentialdifferenzen zwischen Flüssigkeiten und Luft, *Koll. Z.*, 35: 340–342 (1924); Phasengrenzkräfte und Adsorption an der Trennungsfläche Luft/Lösung anorganischer Elektrolyte, *Z. physik. Chem.*, 109: 34–48 (1924).

[26] Kruyt, H. R., and Robinson, C., On Lyotropy, *Proc. Koninklijke Akademie van Wetenschappen*, Amsterdam, 29: 1244–1250 (1926).

was only 56.6 per cent of the water solubility. They suggest that there is an orientation of the dipoles of water in the immediate neighborhood of the ions and that two kinds of orientation are possible. Either the positive end or the negative end of the water molecule may be turned toward the molecules of the solute. Similar orientations of water are postulated to occur on the anions and cations of the electrolytes, and it is the interaction of these three orientations that determines the behavior of a given system.

Büchner [27] and his students, from a study of the effect of ions on a number of systems, measured by a variety of technics, propose an arbitrary "lyotropic number" for the various anions and cations which can be applied to reduce the behavior of the ions to a common denominator. The numbers proposed apparently have no theoretical explanation but are derived empirically from a comparison of the behavior of the ions on experimental systems. For the cations the numbers proposed are Li 115, Na 100, K 75, Rb 69.5, Cs 60, and for anions F 4.8, IO_3 6.25, H_2PO_2 8.3, BrO_3 9.55, Cl 10, NO_2 10.2, ClO_3 10.65, Br 11.3, NO_3 11.6, ClO_4 11.8, I 12.5, and CNS 13.25. A remarkable parallelism is shown between these numbers and the heats of hydration of the ions. For the anion series the relationship is:

$$H = 164 - 8N \tag{125}$$

and for the cation series:

$$H = 27.25 + 0.73N \tag{126}$$

where H = heat of hydration of the ion;

N = the lyotropic number.

Briggs [28] summarized the literature in regard to the effect of ions on electrokinetic potentials, and reached the conclusion that the lyotropic series is dependent not only on ionic mobility and hydration, but also on a number of other factors, only a few of which can be quantitatively evaluated at the present time. Among these factors is the ζ-potential. Figure 88 shows changes in the electrokinetic potential for a series of chloride solutions against a cellulose membrane, as measured by streaming potential methods. The initial increase in the negative potential of a cellulose surface, when small amounts of the chlorides of Li, Na, K, and Cs are added, is probably due to a greater

[27] Büchner, E. H., Quantitative Beziehungen in den lyotropen Reihen, *Kolloid Z.*, 75: 1–9 (1936); (with Voet, A., and Bruins, E. M.) Lyotrope Zahlen und Ionen-eigenschaften, *Proc. Acad. Sci. Amsterdam*, 35: 563–569 (1932); (with Büchner de Gruiter, C. S.), Die lyotrope Kationenreihe bei hydrophilen Kolloiden, *Kolloid Z.*, 76: 173–175 (1936). *Cf.* also Voet, A., Zur numerischen Festlegung der lyotropen Reihe der einwertigen Kationen, *Kolloid Z.*, 78: 201–204 (1937).

[28] Briggs, D. R., The ζ-Potential and the Lyotropic Series, *J. Phys. Chem.*, 32: 1646–1662 (1928).

adsorption of the anion than of the cation. At slightly higher concentrations a relatively greater adsorption of the cation takes place, and the potential is decreased. The divalent ions strontium and barium, are more strongly adsorbed throughout the entire range of concentrations than the chloride ion. However, they are not sufficiently adsorbed in the concentrations studied, to reduce the potential to zero. The thorium ion was the only ion which reversed the charge on the cellulose interface.

In discussing the studies, Briggs states that "The magnitude of the ζ-potential at an interface, therefore, does not necessarily parallel in value the adsorption capacity of that interface. It is only one of the forms in which free surface energy may be present and is, therefore, no more important than is surface tension, or any other condition of strain in which the surface energy may be able to exist, in defining the free energy content of the interface phase of a system. In order to completely define the conditions in the interface, the magnitude of all forms in which surface energy can exist must be determined. Adsorption of some substance may take place to a large degree without appreciably altering the magnitude of some forms of free surface energy. For example, it is conceivable that the free energy as exhibited by interfacial tension may be lowered out of all proportion to any corresponding change in the free surface energy represented by the electrokinetic potential. Other substances may affect all forms of the energy when they are adsorbed. Some substances may be adsorbed to cause a decrease in the total free energy of the surface layer, but at the same time increase the amount of free surface energy existing in some particular form."

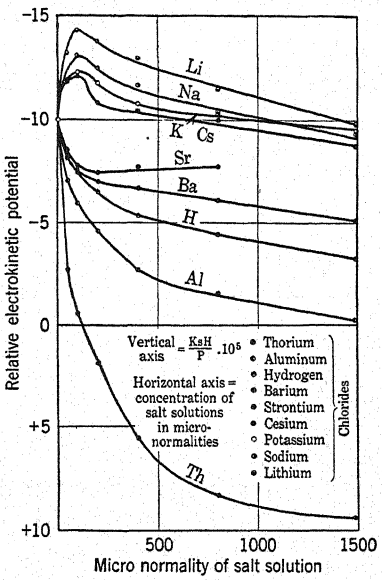


FIG. 88.—Showing a lyotropic series of ions as determined by measuring the electrokinetic potentials at a cellulose-salt solution interface. (Data of Briggs.)

The behavior of any ion will depend not only upon the concentration of that ion but also upon the nature of the system and upon the type of reaction which is being studied. This explains why one author will give a certain lyotropic series as illustrative of the effect of ions on viscosity, and another worker will give a very similar but somewhat

different series for the effect of the same ions in flocculating a sol or in causing peptization. Each worker was securing results which indicated the behavior of ions in the system which he was studying, but such behavior would be slightly altered if other and different factors were introduced.

When organic acids are adsorbed on charcoal [29] a definite lyotropic series could be constructed from the adsorption data. However, the adsorption in this case is apolar,[30] *i.e.*, there is an adsorption of the undissociated molecule, and the magnitude of the electrical charge at the interface is not altered. Accordingly no lyotropic series is evident from electrokinetic studies.

The peptizing behavior of various inorganic salts on wheat flour proteins shows a very pronounced lyotropic series, *e.g.*, 1.0 *N* solution KF peptized on the average 13.07, KCl 22.7, KBr 37.22, and KI 63.89 per cent of the wheat flour proteins.[31, 32] Carpenter [33] found a similar ion series of I > Br > Cl> and Li > Cs > Rb> Na for the effect of the alkali halides on the optical rotation of gelatin in the gel state. The cation effect was greater than the anion effect. The bearing of peptization studies on problems of protein chemistry will be discussed later, it being sufficient to point out here that, *within a series of solutions of equal ionic concentration, there will be found very marked differences in the behavior of such solutions toward protein systems, and as a matter of fact toward all biocolloids.*

The removal of an ion from an interface may often be brought about by adding another ion to the system. Seth [34] pointed out that adsorption is the predominant feature in the interaction between the accumulative poisons, such as lead, arsenic, copper, and mercury, and proteins of the body. The heavy metals are selectively adsorbed by the body proteins and tend to alter the nature of the proteins until finally sufficient quantities are accumulated to cause death. We have already indicated (p. 227) that these same ions are the chief metallic poisons of catalytic surfaces.

[29] Linner, E. R., and Gortner, R. A., *loc. cit.*

[30] Frampton, V. L., and Gortner, R. A., *loc cit.*

[31] Gortner, R. A., Hoffman, W. F., and Sinclair, W. B., Physico-Chemical Studies on Proteins III. Proteins and the Lyotropic Series, Colloid Symposium Monograph, Vol. V, pp. 179–198 (1928).

[32] Gortner, R. A., Hoffman, W. F., and Sinclair, W. B., Zur Kenntnis der Proteine und der lyotropen Reihen, *Kolloid Z.*, 44: 97–108 (1928).

[33] Carpenter, D. C., The Influence of Salts on the Optical Rotation of Gelatin. I., *J. Phys. Chem.*, 31: 1873–1879 (1927); (with Kucera, J. J.), The Influence of Salts on the Optical Rotation of Gelatin. II., *J. Phys. Chem.*, 35: 2619–2626 (1931); (with Lovelace, F. E.), The Influence of Neutral Salts on the Optical Rotation of Gelatin. III. Effect of the Halides of Lithium, Sodium, Rubidium and Cesium, *J. Am. Chem. Soc.*, 57: 2337–2342 (1935); IV. Rotatory Dispersion of Gelatin in Sodium Iodide Solutions, *J. Am. Chem. Soc.*, 57: 2342–2346 (1935).

[34] Seth, Trilok Nath, Adsorption and Mechanism of Poisoning, Pt. I. Irritant Poisons, *Biochem. J.*, 17: 613–621 (1923).

Carder and Coffindaffer [35] argued that, if mercury were adsorbed by the proteins, it should be possible to replace mercury by using an innocuous electrolyte. Thirty-eight dogs were given a fatal dose of 20 mg. $HgCl_2$ per kilo of body weight. Fifteen of these dogs received no further treatment and all died within 1 to 6 days. The 23 remaining dogs received periodically intravenous and intraperitoneal injections of an 0.8 per cent solution of NaCl which were repeated daily until either death or apparent recovery occurred, one month being selected as the time limit, and 19 of the 23 survived.

We have already discussed the apparent lyotropic series of organic radicals which Glick and King found as inhibiting liver esterase, and the effect which others have observed of organic radicals as influencing the electrokinetic potential. Apparently therefore lyotropic series behavior is not strictly limited to ionic effects but seems also to be an expression of the polarity of molecules.

ELECTRICAL PRECIPITATION.—A discussion of the precipitation of suspended particles upon electrified surfaces can be justified at this point because the same basic principles hold for such precipitation as for precipitation by means of electrolytes.

Cottrell [36] has discussed the general theory which is involved and has devised apparatus for satisfactorily causing the precipitation of suspended particles. He notes that "If we bring a needle point connected to one side of a high potential direct current line opposite to a flat plate connected to the other side of the line we find that the air space between becomes highly charged with electricity of the same sign as the needle point irrespective of whether this is positive or negative, and any insulated body brought into this space instantly receives a charge of the same sign. If this body is free to move, as in the case of a floating particle, it will be attracted to the plate of opposite charge and will move at a rate proportional to its charge and the potential gradient between the point and plate."

In "Cottrell precipitation," a high-tension current is applied to chains or rods hung in the stack, and the particles suspended in the gas, becoming electrified, are later precipitated upon plates having the oposite electrical charge, in the same way that two oppositely charged colloidal particles would attract and neutralize each other. The method has been applied not only to gases from smelters [37] but likewise to the

[35] Carder, J. R., and Coffindaffer, R. S., The Value of Forcing Fluid in the Treatment of Mercuric Chloride Poisoning, *J. Am. Med. Assoc.*, 81: 448–450 (1923).

[36] Cottrell, F. G., The Electrical Precipitation of Suspended Particles, *J. Ind. Eng. Chem.*, 3: 542–550 (1911); *cf.* also *J. Ind. Eng. Chem.*, 11: 147–153 (1919).

[37] At Anaconda, Montana, in a stack 72 feet in diameter at the base and 572 feet high, there are four batteries of Cottrell precipitators which require the current from 16 d-c. generators at 62,500 volts. Seventy-six tons of dust with an As_2O_3 content of 42 per cent are collected daily.

dusts from cement plants.[38] In a single cement plant at Riverside, California, approximately 350,000 tons of dust were collected by means of the Cottrell process during a twelve-year period. In this particular installation the treatment was necessary because the cement dust, settling on the citrus groves, interfered with normal photosynthesis. Similar installation has also been used in the breaking of emulsions, particularly natural emulsions of crude petroleum and salt water.[39, 40]

McClendon[41] constructed a laboratory precipitator to avoid the loss of dust when relatively large quantities of grains or other foodstuffs are burned in the process of analyzing them for iodine content. Although the gases of combustion were passed several times through solutions, McClendon observed that solid particles were still present in the gases, and feeling that iodine might be lost, he passed the gases through a Cottrell precipitator. Even at a high velocity of gas flow, the suspended particles precipitated practically quantitatively. Undoubtedly there are many occasions in the laboratory where similar technic could be employed advantageously.

[38] Schmidt, W. A., Electrical Precipitation in Retrospect, *Ind. Eng. Chem.*, 16: 1038–1041 (1924).

[39] Sherrick, J. L., Oil-Field Emulsions, *J. Ind. Eng. Chem.*, 12: 133–139 (1920).

[40] Speed, B., An Appreciation of Dr. Cottrell, *J. Ind. Eng. Chem.*, 11: 153–154 (1919).

[41] McClendon, J. F., The Determination of Traces of Iodine. I. *J. Am. Chem. Soc.*, 50: 1093–1099 (1928).

CHAPTER IX

GELS AND THE WATER RELATIONSHIPS OF THE HYDROPHILIC COLLOIDS

Gels may be defined as more or less rigid colloid systems. Many gels differ from lyophilic sols only in concentration or in degree of dispersion, *i.e.*, a sol may in some instances be transformed into a gel by increasing the content of the disperse phase or may spontaneously pass into a gel by an aggregation of the micelles. Conversely a gel may pass into a sol by the mere process of the peptization of the disperse phase. In the true gels, characteristic of the lyophilic colloids, the gel $\rightleftharpoons$ sol transformation is more or less readily reversed. For example, with gelatin, temperature and hydrogen-ion concentration in a large measure determine whether a given system will be a sol or a gel. Similarly, a starch-water suspension may be converted into a true sol at an elevated temperature and the system will set to a gel when the temperature is lowered.

Coagula produced by the addition of electrolytes to lyophobic systems are often referred to as gels. Such coagula, however, cannot be reconverted into sols without rather drastic peptization treatment.

In apparent viscosity or plasticity, gels may vary from a very thin "jelly," which will flow under rather slight pressure, to rigid structures, such as wool or cotton fibers, filter paper, horn, hair, etc., and the properties of the systems vary with their physical structure.

Undoubtedly gels have a structure; two general views have been put forward as to this structure. Bütschli maintained that gels have a more or less honeycomb structure, the disperse phase forming a network of cell-like walls, the interior being filled with the dispersions medium. Nägeli, on the other hand, suggests a brush-heap structure, in which minute ultramicroscopic fibrils are interlaced throughout the system and the dispersions medium is held within the fibrillar mass by capillary forces in the same way that a crystalline mass of tyrosine or caffeine possesses a certain degree of rigidity.

The evidence appears to be entirely in favor of the brush-heap structure. As little as 0.2 per cent of dibenzoyl-*l*-cystine forms a relatively rigid hydrogel,[1] and even 0.1 per cent forms a soft gel. Ultramicroscopic studies indicate that these gels are of a brush-heap structure, formed by the interlacing of relatively long crystals of di-

[1] Gortner, R. A., and Hoffman, W. F., An Interesting Colloid Gel, *J. Am. Chem. Soc.*, 43: 2199–2202 (1921).

benzoyl-*l*-cystine, the crystals being so thin as to have no apparent cross section. Dibenzoyl-*l*-cystine is not hydrophilic and does not crystallize with water of crystallization. The extreme minuteness of the crystal fibrils apparently favors an intricate network immeshing the dispersions medium. In spite of the fact that dibenzoyl-*l*-cystine does not have associated with it water of crystallization, it must nevertheless be regarded as a polar substance, owing to the presence of amino groups, carboxyl groups, and the —S—S— linkage. We would accordingly expect it to attract water, a polar liquid.

Kraemer [2] used the motion-picture camera as an adjunct to ultramicroscopic studies of gelatin gels and dibenzoyl cystine gel during the gelling process. The gelatin gels were also studied during the melting process. By introducing small mercury particles into the gels, he showed that the resistance of the gel to Brownian movement was not uniform, but that in a dibenzoyl cystine gel the space between the fibrils was occupied by the dispersions medium which appeared to have essentially the same viscosity as water in bulk. In gelatin gels, channels appeared to exist where the Brownian movement of the mercury particles was but slightly impeded, and at other points definite resistance to Brownian movement could be demonstrated. Although no fibers were visible at these points of apparent resistance to the Brownian movement of the mercury particle, it appears as if fibers having approximately the same refractive index as water may well have been present in these areas.

Furthermore, if Bütschli's view were correct, one would expect to find a resistance to the diffusion of a given material or the resistance to the passage of an electrical current through a dilute gel. However, such differences, if they do exist, are extremely slight.

The author has observed many instances where a plant sap, when expressed from leaves, was, though relatively viscous, definitely not a gel. The specific electrical conductivity of such a system remained constant during and following complete gelation, and in a number of instances the gel structure underwent contraction with the squeezing out of a clear fluid and the separation of a distinct "clot" of organic material, the liquid which was squeezed out of the clot being perfectly limpid and still possessing the same specific conductivity as was possessed by the freshly expressed sap or by the gel. The specific electrical conductivity of blood likewise undergoes practically no change during the clotting process.[3] It is only when a system possesses a relatively high percentage of disperse phase that the specific electrical conductivity decreases even slightly on passing from a sol to a gel.

[2] Kraemer, E. O., Studies with the Kinoultramicroscope, Colloid Symposium Monograph, Vol. II, pp. 57–69, Chemical Catalog Company, New York (1925).

[3] Gelfan, S., and Quigley, J. P., Conductivity of Blood during Coagulation, *Am. J. Physiol.*, 94: 531–534 (1930).

Many of the earlier papers on gel structure were based on observations of sections of gels which had been studied by the usual microscopic technic, *i.e.*, the gels had been "fixed" and hardened by the usual biological laboratory reagents, after which they were dehydrated, sectioned, and studied under the microscope. The fixing and hardening reagents of the cytological laboratory are in reality reagents which transform the hydrophilic biocolloids into lyophobic systems. Accordingly one of the most important phases of the biocolloid system, namely the water, is eliminated at an early stage in the process, and undoubtedly rearrangements take place between the organic portions when their affinity for water is destroyed. It may well be that the distribution of the organic colloid particles is entirely different in the original gel from what it appears to be in the fixed, dehydrated, and stained sections which are ultimately studied. The honeycomb structures originally observed by Bütschli appear to have been artifacts.

The tendency of a given system to gelate is in direct proportion to the affinities which exist between the disperse phase and the dispersions medium. If the dispersions medium is strongly attracted by the disperse phase, so that the disperse phase becomes highly solvated, the particles of the disperse phase become surrounded by "shells" of the dispersions medium, the apparent viscosity of the system is greatly increased, until finally the system becomes more or less rigid and a gel results. We are dealing in such instances with surface forces where electrical attractions, interfacial tension forces, and molecular orientation are all operative.

THE FORMATION OF GELS.—The general methods for the formation of gels may be divided into four groups.

(*a*) *The Allowing of a Sol to "Set" after a Change in Temperature, Hydrogen-ion Concentration, Electrolyte Content, etc.*—Typical examples are the formation of a gelatin gel from a gelatin sol by the lowering of the temperature, or the formation of a silicic acid gel by the addition of an acid to sodium silicate. In the case of gelatin a considerable period of time must elapse before a rigid gel is obtained. In the case of sodium silicate the gel forms within a relatively few minutes.

(*b*) *The Dialyzing Out of a Peptizing Agent from a Sol.*—If a concentrated solution of ferric chloride is added to an equivalent solution of sodium arsenate or of phosphoric acid, clear, limpid sols will result. If these sols are dialyzed until the excess electrolytes have been removed, gels will be obtained.

(*c*) *The Solid Imbibing the Dispersions Medium.*—This is the simplest method of gel formation and is typified by the swelling of gelatin in water, the swelling of rubber in organic solvents, and is characteristic of most of the biocolloids.

(*d*) *The Addition of a Third Phase to a System.*—Cellulose nitrate, for example, is not appreciably peptized by either ethyl alcohol or by ethyl ether, but is readily peptized by a mixture of the two. In some instances the addition of a small amount of a third material may produce relatively great changes in a given system. Thus,[4] traces of water in systems of turpentine and sodium stearate, potassium stearate, and sodium oleate have a very great effect upon the gelating properties of such systems, in the case of the sodium stearate favoring the formation of gels, and in the case of sodium oleate inhibiting gel formation. Figure 89 shows the maximum amount of turpentine which would be taken up and held in the form of a gel by 0.05 gram of the disperse phase in the presence of varying amounts of water. Molecular orientation seems to afford the only explanation for this behavior.

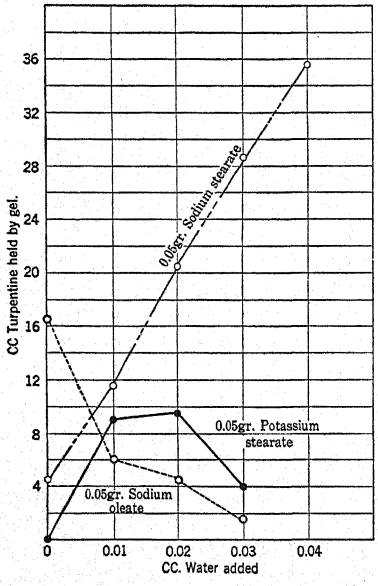


FIG. 89.—Showing the influence of minute traces of water on the formation of turpentine-soap gels. (Data of Holmes and Maxson.)

THE SOLVATION OF LYOPHILIC COLLOIDS.—The lyophilic colloids are distinguished from the lyophobic colloids by an affinity for the dispersions medium. In some instances at least molecules of the dispersions medium appear to penetrate within the structure of the disperse phase and loosen the secondary valence bonds which hold the molecules of the micelle together. Carried far enough this would eventually yield a molecularly dispersed solution. For many lyophilic colloids, dispersion does not progress to the point of a true solution. In many instances the dispersions medium appears to be also oriented in a "shell" surrounding the disperse phase. Solvation is easily followed by changes in viscosity. In Fig. 12 is shown the very rapid fall in viscosity which takes place in an acidulated flour-water suspension when traces of inorganic salts are added. In the particular experiments in question, the maximum viscosity reached yielded a fairly coherent gel and within, at the most, two or three seconds after the addition of the mag-

[4] Holmes, H. N., and Maxson, R. N., The Influence of a Second Liquid upon the Formation of Soap Gels, Colloid Symposium Monograph, Vol. V, pp. 287–300 (1928).

nesium sulfate, the viscosity had fallen to practically that of the dispersions medium. It would appear as if a large fraction of the dispersions medium had been oriented on the surface of the protein micelles, by electrical forces, and that this oriented water shell had been released by the neutralizing effect of the inorganic salt.

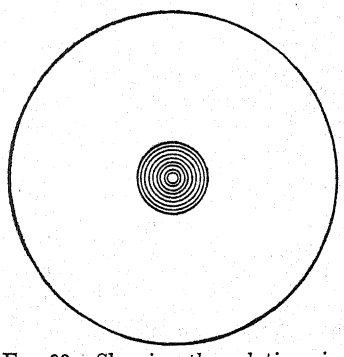


FIG. 90.—Showing the relative size of colloidal particles of the dyestuff, "Geranin G," and the associated water shell. (Data of Liepatoff.)

Liepatoff [5] estimated the thickness of the water shell which was adsorbed onto the surface of a colloidal particle of the dyestuff, Geranin G, and concluded that the radius of the dyestuff micelle is approximately 4.9×10^{-8} cm. and that this is surrounded by a water shell 17.6×10^{-8} cm. in thickness. Figure 90 shows such a dyestuff particle and the adsorbed water shell in their proportionate relationship. It is evident from such a diagram that relatively large viscosity changes may be brought about by relatively small amounts of the disperse phase, inasmuch as every added increment of the disperse phase combines with a much larger amount of the dispersions medium, removing it from a mobile to a more or less fixed state.

That there is a certain packing of the molecules of the dispersions medium in the shell surrounding the micelles is evidenced by the fact that a volume change occurs in the formation of a gel, so that the volume of the gel is less than the volume which would be occupied by the dry disperse phase and the pure dispersions medium. Various workers have studied this problem. Chick and Martin [6] reached the conclusion that the density of protein micelles in sols is from 5 to 8 per cent greater than the density of protein in the dry state. Svedberg [7] in a more exact series of studies finds a contraction in the system of about 54 cu. mm. per gram of gelatin dissolved in water at 35°. The greatest initial contraction occurs with the initial increments of water which are added. The contraction increases with a decreased temperature, which apparently indicates an adsorption reaction. It is doubtful that there is in reality an increase in the density of the gelatin micelles, the more probable explanation being that the water molecules

[5] Liepatoff, S. von, Über die Viskosität und Hydration von Farbstofflösungen, *Kolloid Z.*, 39: 230–236 (1926).

[6] Chick, H., and Martin, C. J., Die Dichte und das Lösungsvolumen einiger Proteine, *Kolloid Z.*, 12: 69–71 (1913).

[7] Svedberg, The., Density and Hydration in Gelatin Sols and Gels, *J. Am. Chem. Soc.*, 46: 2673–2676 (1924).

are oriented on the surface of the protein micelles, and in this oriented state they occupy a smaller volume than the water molecules which are in the bulk of the liquid.

That gels possess a structure and that there are attraction forces between the disperse phase and the dispersions medium is further evidenced by the work of Holmes, Kaufmann, and Nicholas.[8] They allowed silica gels to set in test-tubes of the same length but of varying diameters. When the tubes containing the gels were tapped, sound-producing vibrations were set up which were related not to the length of the column of the gel but rather to the diameter of the gel column. A vibration frequency of 341 per second was found for a column of gel 34 mm. in diameter, increasing to 640 vibrations per second for a column 23 mm. in diameter, and further increasing to a frequency of 1152 vibrations per second when the column was only 15 mm. in diameter. An increase in the concentration of the silica gel increased the pitch of the vibration. These vibrations were due to a rigidity of gel structure. If the test-tubes were previously greased so that the gel could not adhere to the glass, no vibration was observed. When silica gels in a state of tension similar to the above "musical" gels are allowed to stand for any considerable period of time, they are usually torn apart by their own internal stresses, in some instances with such force as to shatter the wall of the tube enclosing them. In such gels the entire system reacts as a rigid solid, indicating that the disperse phase has bound all or nearly all of the dispersions medium.

Van Bemmelen[9] in his classic study of silica gels proved that the water which is present is not water of constitution, for he could secure no definite evidence of the presence of any hydrates of SiO_2. The attraction of water for an SiO_2 surface appears to be due to the fact that an SiO_2 surface is a polar surface and water is a polar liquid. He did show that the amount of water retained by a silica gel was dependent on the past history of the gel. He found that the dehydration curve of silica gel was a continuous process but was not an equilibrium. A silica gel which has been dehydrated to a certain point will again take up water if placed in a suitable environment, but the rehydration curve will not follow the dehydration path. Figure 91 shows the familiar dehydration-hydration curves of silicic acid gels as found by van Bemmelen. The arrows pointing downward indicate the dehydrating processes, the arrows pointing upward indicate the hydrating processes. As a rule, gels which have been partially dehydrated have a lowered capacity for reimbibing water. This is characteristic not only of silica gels but of all, or nearly all, of the organic gels. *The aging of a gel is similarly characterized by a decrease in*

[8] Holmes, H. N., Kaufmann, W. E., and Nicholas, H. O., The Vibration and Syneresis of Silicic Acid Gels, *J. Am. Chem. Soc.*, 41: 1329–1336 (1919).

[9] van Bemmelen, J. M., Die Absorption, Theodor Steinkopff, Dresden (1910).

the intensity of the forces with which the dispersions medium is held. This is another point at which the *time* factor enters into colloidal behavior and is usually one of the most striking examples of hysteresis.

The tenacity with which water is held upon the surface of aluminum oxide has already been referred to. An aluminum oxide gel which has been heated to 700 to 750° is one of the most efficient drying agents known and is used in organic combustions in place of calcium chloride or P_2O_5. Johnson[10] states that "1 gram of Al_2O_3 can practically completely absorb all moisture from approximately 10 liters of air saturated with water vapor at 18°." Aluminum oxide which is perfectly anhydrous will not act as an efficient adsorbent for water, for

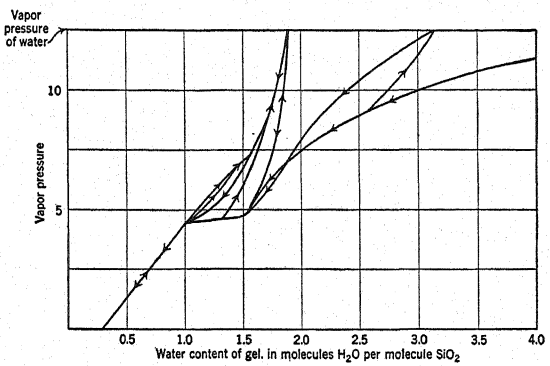


FIG. 91.—Hydration $\rightleftarrows$ dehydration curves of silica gel. (Data of van Bemmelen.)

the adsorbing properties depend upon an aluminum gel from which not all the original water has been removed.

The terms, solvation, hydration, and imbibition, are used interchangeably in referring to the process by which water is taken up by the biocolloids.

METHODS FOR MEASURING IMBIBITION.—Three general methods have been used for the measurement of imbibition by lyophilic colloids: (1) the swelling of granules of the lyophilic colloid when placed in water, (2) the increase in weight of the lyophilic colloid when placed in water or in atmospheres of varying humidity, and (3) changes in viscosity of the system containing the lyophilic colloids.

The method for increase in volume was used largely by Martin Fischer in his studies of the hydration of fibrin and other animal proteins. A given amount of the protein is placed in test-tubes of a uniform bore, and the various solutions whose effect on imbibition it is desired to study are added. At varying intervals of time the height of the swollen column of protein is measured, and the amount of imbibi-

[10] Johnson, F. M. G., Alumina as a Drying Agent, *J. Am. Chem. Soc.*, 34: 911–912 (1912).

tion which has taken place is calculated from the height of the swollen column.

Hofmeister was probably the first to use the increase-in-weight method. A sheet of gelatin or some similar lyophilic colloid is placed in the liquid to be studied and from time to time is removed and weighed. The increase in weight plotted against time will give an imbibition curve. This method has been used in studying the quality of wheat-flour proteins.[11, 12]

Wo. Ostwald [13] notes among other factors that viscosity increases with increasing solvation of lyophilic colloids. Gortner and Sharp [14, 15] and Lüers and Ostwald [16] almost simultaneously applied the viscosity method to the study of wheat-flour proteins, and in a later paper Lüers and Schneider [17] carried out a comparative study in which Hofmeister's method of weighing and Fischer's method of volume change were compared with the viscosity method. They reached the conclusion that all three methods are equally suitable for measuring hydration capacity. The viscosity method permits of greater ease of manipulation and of a considerably higher degree of accuracy. Accordingly it seems the preferable method to employ whenever experimental conditions permit of its use. We have already discussed equations (6), (7), and (8) by means of which volume changes of the disperse phase may be followed.

IMBIBITION PRESSURE.—Many organic colloids show a great affinity for certain liquids and take up such liquids against relatively enormous pressures. The pressure against which such a colloid will imbibe a liquid, or conversely the pressure which is required to force the dispersions medium out of a gel, is known as the imbibition pressure. Imbibition pressures should not be confused with osmotic pressure. In many instances the magnitude of imbibition pressure greatly exceeds that of osmotic pressure. For example, seeds of *Xanthium glabratum* containing 8 to 9 per cent of water will, through imbibition pressure, withdraw water from a saturated solution of lithium chloride which

[11] Upson, F. W., and Calvin, J. W., On the Colloidal Swelling of Wheat Gluten, *J. Am. Chem. Soc.*, 37: 1295–1304 (1915).

[12] Gortner, R. A., and Doherty, E. H., Hydration Capacity of Gluten from "Strong" and "Weak" Flours, *J. Agr. Research*, 13: 389–418 (1918).

[13] Ostwald, Wo., Über die Bedeutung der Viskosität für das Studium des kolloiden Zustandes, *Kolloid Z.*, 12: 213–222 (1913).

[14] Gortner, R. A., and Sharp, P. F., The Physico-Chemical Properties of Strong and Weak Flours. III. Viscosity as a Measure of Hydration Capacity and the Relation of the Hydrogen Ion Concentration to Imbibition in the Different Acids, *J. Phys. Chem.*, 27: 481–492 (1923).

[15] Sharp, P. F., and Gortner, R. A., Viscosity as a Measure of Hydration Capacity of Wheat Flour and Its Relation to Baking Strength, Technical Bulletin 19, Minnesota Agricultural Experiment Station, 119 pp. (1923).

[16] Lüers, H., and Ostwald, Wo., Beiträge zur Kolloidchemie des Brotes V. Die kolloide Quellung des Weizenklebers, *Kolloid Z.*, 27: 34–37 (1920).

[17] Lüers, H., and Schneider, M., Zur Messung der Solvatation (Quellung) in Kolloiden, *Kolloid Z.*, 28: 1–4 (1921).

has an osmotic pressure of approximately 965 atmospheres,[18] although the salt content of the seed is sufficient to account for only a few atmospheres of osmotic pressure. Similarly, if a sheet of dried gelatin is placed in a saturated solution of sodium chloride, water will be withdrawn by imbibition forces against the osmotic pressure of the sodium chloride solution, and sodium chloride will crystallize out in the solution. The sap of plants in salt marshes or alkali flats may reach an osmotic pressure as high as 172 atmospheres[19] (14.4° depression of freezing point), but such plants are not characteristic of the more extreme xerophytes, such as the cacti, where the osmotic

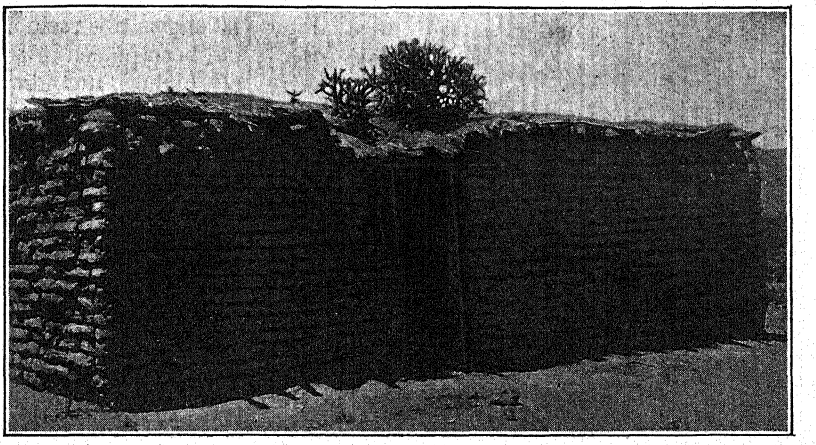


FIG. 92.—*Opuntia* sp. growing in 3 inches of soil on the roof of an adobe house near Tucson, Arizona. Annual rainfall about 10 inches.

pressure plays a minor role and imbibition pressure becomes all important.

Figure 92 shows a cactus (*Opuntia* sp.) growing in 3 inches of adobe soil on the roof of an Indian hut near Tucson, Arizona, in a region of approximately 10 inches of annual rainfall. The osmotic pressure of such cacti does not exceed 6 or 7 atmospheres as measured by the depression of the freezing point of the sap. Nevertheless, the imbibition pressure of the colloids which are present in the mucilaginous juices is so great as to withdraw water from an extremely dry soil and to resist the desiccating effects of the desert winds throughout long periods devoid of rainfall.

[18] Shull, C. A., Semipermeability of Seed Coats, *Botan. Gaz.*, 56: 169–199 (1913); Imbibition in Relation to Absorption and Transportation of Water in Plants, *Ecology*, 5: 230–240 (1924).

[19] Harris, J. A., Gortner, R. A., Hoffman, W. F., and Valentine, A. T., Maximum Values of Osmotic Concentration in Plant Tissue Fluids, *Proc. Soc. Exptl. Biol. Med.*, 18: 106–109 (1921).

Newton [20] and his co-workers, in July, 1925, removed some stems from the Alberta *Opuntia*, sealed the cut surfaces with grafting wax, and then placed the segments in a desiccator over concentrated sulfuric acid. At the end of six months in an atmosphere of almost zero humidity, the stems had lost less than 10 per cent of the water which they originally contained. At the end of 94 days of desiccation, one of the stems was removed from over the sulfuric acid and placed in another desiccator over a free water surface. Figure 93 shows this stem 54 days after it had been placed in the atmosphere of higher humidity. Two new shoots were already well developed. A change in the relative humidity of the air was sufficient to produce a growth response. The ability of the *Opuntia* to resume growth almost as soon as favorable conditions appear is abundant evidence that the apparently drastic desiccation over sulfuric acid was of no real significance so far as the vital activities of the cactus were concerned but that the imbibitional forces with which the colloids of the stem retained and imbibed water constitute a vital factor in the ability of the plant to grow under extremely adverse conditions.

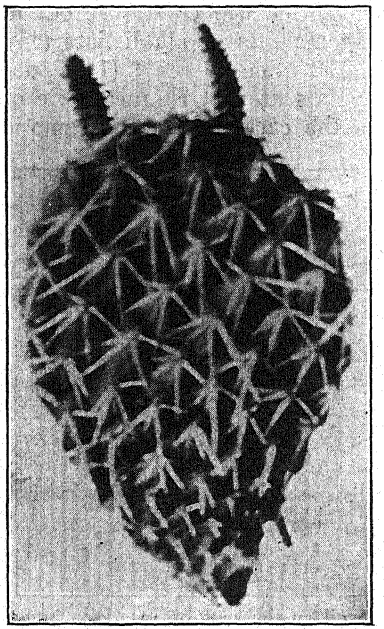


FIG. 93.—*Opuntia* sp. removed from desiccator after 94 days over concentrated H_2SO_4 and placed in another desiccator over pure water. Growth was observed within two weeks of transference. Photograph taken 54 days after transference. (Photograph, courtesy of Dr. Robert Newton.)

Similar examples of the force with which water is taken up or retained by organic colloids are numerous. Starch will still swell against a pressure of 2500 atmospheres if heated in the presence of water. Reinke,[21] in 1879, measured the swelling pressure of dried disks of the sea algae, *Laminaria*, against water. He placed disks of the dried *Laminaria* in a hollow metal cylinder, and above the disks fitted a metallic piston carrying a platform upon which weights could be placed. The disks of dried algae

[20] Newton, R., and Martin, W. McK., Physico-Chemical Studies on the Nature of Drought Resistance in Crop Plants, *Canadian J. Research*, 3: 336–427 (1930).

[21] Reinke, J., Untersuchungen über die Quellung einiger vegetabilischer Substanzen, *Hanstein's botanische Abhandl.*, 4: 1–137, 4 plates (1879).

were then allowed to come in contact with water, and the amount of swelling against known weights was measured by the movements of the piston. When no further swelling took place, a part of the weights could be removed, and swelling again set in, reaching a new equilibrium determined by the weight of the piston. Figure 94 shows the

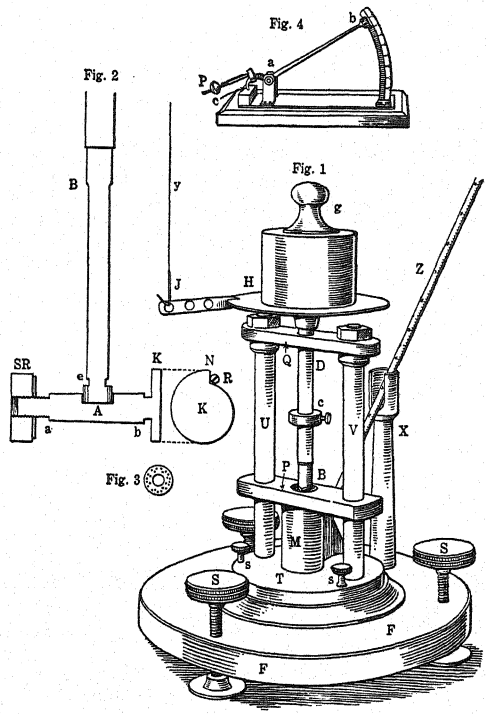


FIG. 94.—Reinke's original apparatus for measuring imbibition pressure.

original apparatus used by Reinke in these pioneer studies of imbibition pressure.

That imbibition pressures are not limited to hydrophilic colloids is evidenced by the work of Posnjak,[22] who studied the swelling pressures of rubber in organic liquids and gelatin in water. He found the same general phenomena in both types of systems.

MacDougal[23] refined the methods of measuring the swelling of biocolloids. The general arrangement of the apparatus is shown in

[22] Posnjak, E., Über den Quellungsdruck, *Kolloidchem. Beihefte,* 3: 417–456 (1912).

[23] MacDougal, D. T., Hydration and Growth, Carnegie Institution of Washington Publication 297, Washington (1920).

Fig. 95. By means of this instrument MacDougal and his co-workers studied various problems involving the swelling of plant colloids, and in his monograph he attempts to correlate the observations with the growth processes.

Various attempts have been made to express in mathematical terms the forces of imbibition. Perhaps the most successful of these have

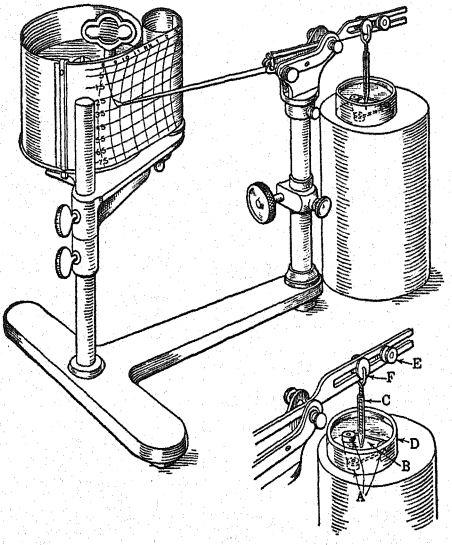


FIG. 95.—MacDougal's auxograph arranged for recording changes in thickness, due to swelling, of trio of sections of plant tissues and of biocolloids. The vertical arm, which is set in position on horizontal arm to give an amplification of 20, rests on a triangle of glass laid on top of the sections. The dish containing the sections rests on an iron cylinder to secure stability, and a weight is placed on the T base of the instrument. The record sheet is ruled to millimeters (not shown) with heavier horizontal lines 1 cm. apart. The heavy curved lines represent hour intervals. The space is ruled to 15-minute intervals (not shown). Height of clock and lever supports adjustable.

been the equations proposed by Wo. Ostwald [24] and Dorothy Jordan-Lloyd.[25] Ostwald's equation is:

$$P = \left(\frac{RT}{M}\right) C + KC^n \qquad (127)$$

[24] Ostwald, Wo., Über Osmose und Quellung disperser Systeme, *Kolloid Z.*, 24: 7–27 (1919); Zur Kenntnis der allgemeineren Solvationsgleichung kolloider Systeme, *Kolloid Z.*, 49: 60–74 (1929); and Über Osmose und Solvatation disperser Systeme, *Z. physikal. Chem.*, (A) 159: 375–392 (1932).

[25] Lloyd, Dorothy Jordan-, The Absorption of Water by Gelatin IV. The Influence of Temperature, *Biochem. J.*, 24: 1460–1471 (1930); VI. The Influence of the Thickness and Original Concentration of the Gel, *Biochem. J.*, 25: 1580–1592 (1931).

where P = the swelling pressure;
C = the concentration of the sol;
M = particle weight;
K and n = constants.

The first part of equation (127) is essentially the true van't Hoff osmotic pressure which increases in direct proportion to the concentration and the absolute temperature. The second portion of the equation is an adsorption isotherm and indicates that the swelling pressure increases disproportionately with an increase in concentration. The constants K and n vary greatly for different colloid systems. A graph of this equation as compared with ordinary osmotic pressure effects is shown in Fig. 96.

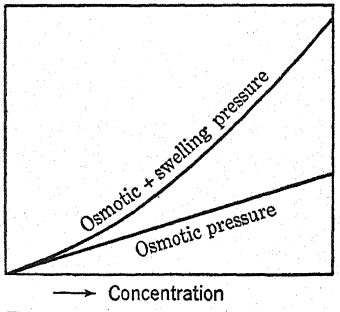


FIG. 96.—A diagrammatic representation of the effect of concentration of lyophilic sols on osmotic pressure and on swelling pressure. (After Wo. Ostwald.)

Miss Lloyd's equation is:

$$S = K_1\left(\frac{1}{\log C}\right) + K_2 \quad (128)$$

where S = the swelling;
C = the original concentration of the gel;
K_1 and K_2 = constants, K_2 equaling some function of K_1.

She found this equation to express rather exactly the swelling of gelatin gels where the original concentration of the gelatin did not exceed 20 per cent. For an individual gelatin gel in salt solutions she found that swelling was independent of the volume of the salt solution but was directly proportional to the logarithm of the concentration of the salt.

$$S = a \log C_1 \quad (129)$$

where C_1 = the concentration of the salt:
a = a constant depending upon the nature of the salt.

As long as the swelling process does not proceed so far as to induce solation, a is independent of temperature. She lists three mechanisms as influencing swelling: (1) true imbibition which she defines as the taking up of the dispersions medium until the gel reaches a preformed structure, (2) osmotic swelling due to unequal solvent pressure in the solution and in the gel, and (3) swelling of hydration characteristic of salt solutions and of acid or alkaline solutions some distance removed from the isoelectric point of the colloid.

COLLOID OSMOTIC PRESSURE.—We have already briefly discussed the osmotic pressure of colloid systems (p. 71), and that discussion is pertinent in the present connection. The colloid osmotic pressure of blood serum and blood plasma has been extensively investigated by many workers. Meyer [26] presents an excellent summary of the entire field covering the literature prior to 1932. He discusses the physicochemical basis of colloid osmotic pressure, methods of measurement, compounds involved, the relationship of colloid osmotic pressure to the behavior of normal and pathological organisms, positive and negative water balance, the action of diuretics, hormones, soporifics, etc. In a study of the colloid osmotic pressure of the body fluids of animals he [27] suggests that it is but an expression of the general organization of the animal and that the values for colloid osmotic pressure fall in a general arrangement in the order of phylogenetic relationships. Table XXXVII lists the colloid osmotic pressures of the bloods of various animals as noted by Meyer.

TABLE XXXVII

"COLLOID OSMOTIC PRESSURE" OF THE BLOOD OF VARIOUS ANIMALS
(DATA OF PAUL MEYER)

Animal	Colloid Osmotic Pressure, cm. H_2O	Animal	Colloid Osmotic Pressure, cm. H_2O
Pigeon	7.5–12	Goat	30–31
Chicken	13.5–15.5	Sheep	29–33.5
Frog	±27.5	Cattle	26–30
Rat	22–29	Horse	22.5–35
Guinea pig	22–28	Man (birth)	±26.5
Rabbit	22–35	Man (mos.)	±32.7
Dog	24–40	Man (adult)	27–42
Cat	24–33	Man (old)	3–5 cm. higher
Hog	32–35		maximum ±55

Colloid osmotic pressure in man appears to be intimately related to the edemas of hemorrhage and nephritis. The loss of proteins from the blood stream reduces the colloid osmotic pressure within the capillaries so that the tissue colloids are able to withdraw water from the blood stream into the tissues. The hydrated tissue colloids then undergo syneresis so that large volumes of water collect within the tissues instead of being excreted normally through the kidneys. Sir

[26] Meyer, Paul, Der kolloidosmotische Druck biologischer Flüssigkeiten, *Ergeb. Physiol.*, 34: 18–111 (1932); *cf.* also Peters, *loc. cit.*

[27] Meyer, Paul, Colloid Osmotic Pressure of the Body Fluids of Marine Animals, *Nature*, 136: 757–758 (1935).

William Bayliss [28] was apparently the first to suggest the intravenous injection of hydrophilic colloids into the blood stream to replace blood proteins which had been lost through hemorrhage or to raise the colloid osmotic pressure of the blood. During the World War intravenous injections of gelatin were used and later intravenous injections of gum acacia. Gum acacia injections are now a routine procedure in most hospitals. Bayliss has made the statement that by use of gelatin and later gum acacia in cases of excessive hemorrhage the lives of at least 20,000 wounded men were saved in the Allied Forces. Meyer illustrates the influence of the blood colloids on the water balance by an experiment in which a 30 per cent solution of sodium arabate in physiological salt solution was intravenously injected into dogs in an amount equivalent to the blood volume. The colloid osmotic pressure increased sharply from 30 cm. H_2O to 46 cm. H_2O but fell in 15 minutes to its original value. A slower fall to 28 cm. H_2O then occurred, the original 30-cm. value being regained only after several hours. Urine secretion stopped completely for the first hour but slowly increased during the next three or four hours and then became excessive. Sodium arabate initially appeared in the urine in large amounts, but then disappeared from the urine and reappeared in the vomit and feces. During the period of urine cessation the skin became dry and harsh, and the animal showed every appearance of being dehydrated.

HEAT OF IMBIBITION.—We discussed in Chapter VII the question of heat of adsorption. Imbibition can be regarded as the adsorption of the dispersions medium, and accordingly the heat of imbibition is only a special example of heat of adsorption. What has been said of heat of adsorption will accordingly apply equally well to heat of imbibition. We have noted that there is a contraction of the system when a gel imbibes the dispersions medium, and we have likewise noted that there is a positive imbibition pressure. The volume contraction is directly related to the imbibition pressure, and, if there is a positive imbibition pressure, there must be an evolution of heat under such pressure. Accordingly one can construct an imbibition curve by following either the volume changes which take place during imbibition or by following an imbibition pressure curve or by following the temperature changes which occur during the imbibition process.

Imbibition should not be confused with solution. The imbibition process is in general characterized by an evolution of heat. On the contrary, the solution process is in general characterized by an absorption of heat. Thus, in gelatin the initial swelling process liberates heat, whereas the final dispersing process absorbs heat. From the heat which is liberated it is possible to reconstruct the imbibition curve.

[28] Bayliss, W. M., Methods of Raising a Low Arterial Pressure, *Proc. Roy. Soc. (London), (B)*, 89: 380–393 (1916); The Action of Gum Acacia on the Circulation, *J. Pharmacol.*, 15: 29–74 (1920).

The curves so constructed are typical adsorption curves, being parabolas when the numerical values are plotted and straight lines when the ordinates and abscissas are logarithmic values.

RATE OF IMBIBITION AND MAXIMUM IMBIBITION CAPACITY.—The determination of the rate of imbibition and of the maximum imbibition capacity is in some instances of the utmost importance in detecting differences in the degree of colloidality of lyophilic colloids.[29] Those lyophilic gels in which the colloidal structure is highly developed normally show a much greater rate of imbibition and a much greater maximum imbibition capacity than characterize gels of the same constituents but of a less perfect colloidal structure.

THE EFFECT OF HYDROGEN-ION CONCENTRATION ON IMBIBITION.—Protein systems show a remarkable effect of hydrogen-ion concentration on rate of imbibition and maximum imbibition capacity. The preliminary work in this field was carried out by Martin Fischer[30] in an attempt to apply colloid studies to pathological problems. Most of Fischer's imbibition curves were drawn with normality of acid concentration as one of the variables rather than hydrogen-ion concentration, because his experiments were made before hydrogen-ion-concentration measurements were a part of the technic of the biochemical laboratory. He noted that the various acids produced very dissimilar curves. The lack of similarity was due to the fact that the various acids with which he worked had very different hydrogen-ion concentrations at equivalent normalities, and had his curves been drawn with respect to a single variable, *i.e.*, the hydrogen-ion concentration, they would all have possessed a similar shape; *cf.* Figs. 34 and 35. Loeb[31] stressed especially the importance of hydrogen-ion concentration as one of the major factors in the swelling of proteins in acid or alkaline solutions.

It would take us too far afield to list all or even the more important papers having to do with the effect of hydrogen-ion concentration on imbibition in protein systems. There seems, however, to be a unanimity of opinion that a minimal degree of swelling occurs in solutions where the protein is in an isoelectric condition. At hydrogen-ion concentrations on either side of the isoelectric point there is a rapid increase in imbibition which reaches a maximum on the acid side at approximately *p*H 2.5–3.0 and on the alkaline side at approximately *p*H 10.5. In contrast to protein systems the carbohydrate gels like

[29] Gortner, R. A., and Doherty, E. H., Hydration Capacity of Gluten from "Strong" and "Weak" Flours, *J. Agr. Research*, 13: 389–418 (1918); Sharp, P. F., and Gortner, R. A., Physico-Chemical Studies of Strong and Weak Flours. II. The Imbibitional Properties of the Glutens from Strong and Weak Flours, *J. Phys. Chem.*, 26: 101–136 (1922).

[30] Fischer, Martin H., Oedema and Nephritis, Third Edition, John Wiley and Sons, New York (1921).

[31] Loeb, J., Proteins and the Theory of Colloidal Behavior, McGraw-Hill Book Company, New York (1922). (*Cf.* also numerous papers in the *Journal of General Physiology*, Vols. 1–7, inclusive.)

agar and cactus mucilage have a maximum imbibition in solutions which are essentially neutral and at hydrogen-ion concentrations differing appreciably from the neutral point there is a decreased imbibition capacity.[32] The imbibition curves of plant tissues can be duplicated by mixtures of carbohydrate and protein, and MacDougal suggests that the curves characteristic of plant tissues are due to a mixture of approximately 9 parts of a polysaccharide, such as agar, and 1 part of a protein. His observation that very dilute solutions of amino acids favor imbibition of his "biocolloids" to a rather remarkable degree remains to be explained.

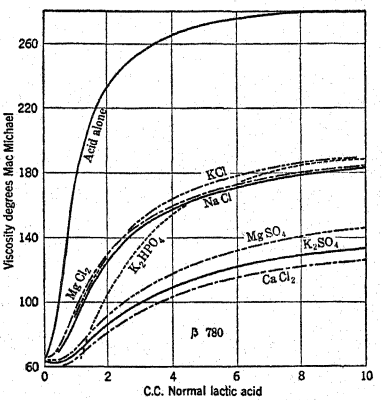


FIG. 97.—Imbibition curves as measured by the viscosity of a 20 per cent flour-in-water suspension made 0.01 *N* with respect to various salts and with the subsequent addition of various amounts of lactic acid. (Data of Gortner and Sharp.)

THE EFFECT OF SALTS ON IMBIBITION.—Neutral solutions of electrolytes influence imbibition in a manner very similar to their effect upon lyophobic sols. Figure 97 shows the decrease in viscosity of an acidulated wheat flour-water suspension to which various salts were added. It will be noted that there is a distinct lyotropic series. The original lyotropic series of Hofmeister was based upon observations of the swelling of proteins in various solutions. Bayliss[33] gives an excellent discussion of the role that colloid chemistry plays in physiology and (pp. 137–143) devotes a considerable part of the discussion to the relation of electrolytes to the biocolloids.

SYNERESIS.—Syneresis may be looked upon as the reverse of imbibition. Heller[34] classifies syneresis under three categories of causes: (1) syneresis of desorption, caused by the particle becoming less hydrophilic with time, *e.g.*, increase in particle size or crystal growth and a decrease in active surface area; (2) syneresis of aggregation, discrete gel particles (geloides) may unite into a denser gel portion and a more liquid phase remaining exterior to the denser gel; and (3) syneresis of contraction, where a gel with a fibrillar structure contracts and the shrinking forces squeeze out the intermicellar liquid. The separation

[32] MacDougal, D. T., Hydration and Growth, Carnegie Institution of Washington Publication 297, Washington (1920).

[33] Bayliss, W. M., Colloid Chemistry in Physiology, Second Report on Colloid Chemistry, pp. 117–154, Brit. Assoc. Advancement Sci., London (1918).

[34] Heller, W., Essai d'une systématique de la synérèse, *Compt. rend.*, 204: 43 (1937).

of serum from a blood clot, the "bleeding" of an agar slant, the liquid which collects around the mold of gelatin in an ice box, the separation of whey when milk sours, or in the cheese manufacturing process, the "sweating" of bread, "leaky" butter, the separation of liquid from lean meat when heated, and the serum that exudes from a wound or blister are all typical examples of the phenomenon of syneresis.

Wo. Ostwald states that syneresis is one of the most characteristic properties of a gel. The liquid which exudes from the gel is not pure water but rather is a dilute sol, the composition of which is determined by that of the original gel.[35]

Inasmuch as protoplasm is a true colloid system, it must "secrete" when it contracts. The liquid in the contractile vacuole is liquid of syneresis. Any change in the surface properties of the biocolloids shifts their water balance either in the direction of increased imbibition or in the direction of syneresis. The change in surface properties need not be great to bring about very appreciable changes in the water-holding capacity. The secretions of the ductless glands are in all probability the result of syneresis, and for certain of these colloids at least a nerve impulse is sufficient to alter the surface properties and to bring about an increased secretion of hormones.

The Aging of Gels.—The *time* factor affects all colloid systems but is particularly noticeable in gels. The micelles may aggregate into larger units, or crystal growth may be a factor, and the larger particles may grow at the expense of the smaller ones, owing to an unequal distribution of surface energy forces. With the biocolloids there is a decrease in imbibition capacity with age. Probably the changes involved in senescence are in a large measure changes characteristic of aged colloid systems. The older leaves on a tree are characteristically more lignified and contain a lower content of colloids in the sap which can be expressed than the leaves which are just unfolding. Old tissues, both of plants and of animals, are in general less highly hydrated than the younger tissues. The walls of the blood vessels of older animals contain a higher proportion of dry matter, a higher proportion of inorganic constituents, notably calcium, and a much lower proportion of water, than walls of the blood vessels of young animals.

The body tissues can be looked upon as examples of colloid gels, the behavior of which may be expected to be intimately related to the gel structure and the water content. In the hardening of the arteries (arterio sclerosis) of old age, we have a striking example of the loss of flexibility of the arterial wall which is associated with a higher content of dry matter and a reduced imbibition capacity. Thoenes[36] has like-

[35] Healy, D. J., The Exudate from Nutrient Agar Slants, The So-Called Water of Condensation, *J. Bact.*, 12: 179–180 (1926).

[36] Thoenes, F., Untersuchungen zur Frage der Wasserbindung in Kolloiden und tierischen Geweben, *Biochem. Z.*, 157: 174–186 (1925).

wise shown that the muscle tissues of dogs and guinea pigs have a progressively lowered imbibitional capacity as the animal becomes older. If the problems of rejuvenescence are ever solved, they will be solved very largely through colloid chemical studies designed to bring about an increased, or to maintain a high, imbibition capacity of the tissue colloids.

THE ROLE OF WATER IN THE LIVING ORGANISM.—Inasmuch as living organisms are very largely composed of water, a study of the gels which comprise the living organism is extremely important.

TABLE XXXVIII

COMPOSITION OF THE HUMAN BODY

Elementary Composition		Group Composition	
	Per Cent		
Oxygen	66.0		
Carbon	17.5		
Hydrogen	10.2		
Nitrogen	2.4		
Calcium	1.6		Per Cent
Phosphorus	0.9	Water	65.0
Potassium	0.4	Protein	15.0
Sodium	0.3	Fat	14.0
Chlorine	0.3	Salts	5.0
Sulfur	0.2	Other organic compounds	1.0
Magnesium	0.05		
Iron	0.004		
Iodine, Fluorine, Silicon, Manganese, Arsenic, etc.	Present		

Table XXXVIII gives the approximate elementary composition of the human body, as well as the relative proportion of water, protein, fat, salts, and other organic compounds. It will be noted that the greater percentage of the human body is composed of water. This is probably true of every living organism. In some organisms, as the jellyfish, only an insignificant fraction is composed of organic material, as little as 1 per cent of the jellyfish being organic matter. Figure 98 illustrates what a small proportion of dry matter enters into the composition of a jellyfish. In this figure the "bell" of a jellyfish has been dried down upon the page of a magazine and the print can be rather readily seen through the dry material of what was formerly a living organism. It has been said that "the sea ebbs and flows through a jellyfish," which is essentially saying that the water of a jellyfish structure plays no particular function. Undoubtedly this is incorrect.

The many striking and unusual results which one obtains in dealing with dilute gels of the lyophilic colloids cannot be duplicated when one works with the dry materials, and the jellyfish must be thought of as a biological system in which water plays a role and probably a more important role than any other single constituent.

In most organisms a very considerable part of the growth process is concerned with the building of water into the living structure. For example, a frog's egg, weighing on the dry basis only a few milligrams, can, after fertilization, be placed in a dish of filtered, sterile water and allowed to undergo the process of development. Such an egg will undergo cell division, giving rise at the end of several weeks to a living tadpole which may be as much as 2 cm. in length and weigh several grams. Such a tadpole has never partaken of food other than utilizing the nutrients already present in the original egg and will be found on analysis to contain less dry matter than the original egg, owing to the fact that a certain amount of the organic materials present have been utilized as a source of energy and eliminated as carbon dioxide and water. The increase in weight during these several weeks has all been due to the intake of liquid water which has become "living water," so the tadpole is actually more than 95 per cent water. It would be ridiculous to speak of this organism as being composed of only 5 per cent of vital materials. The water is as much a part of the tadpole as the fats, proteins, etc., which serve to form the gel structure, and the biochemical and biophysical reactions which take place within the cells and tissues of the tadpole are determined probably more by the water which is present than by any or all of the other constituents.

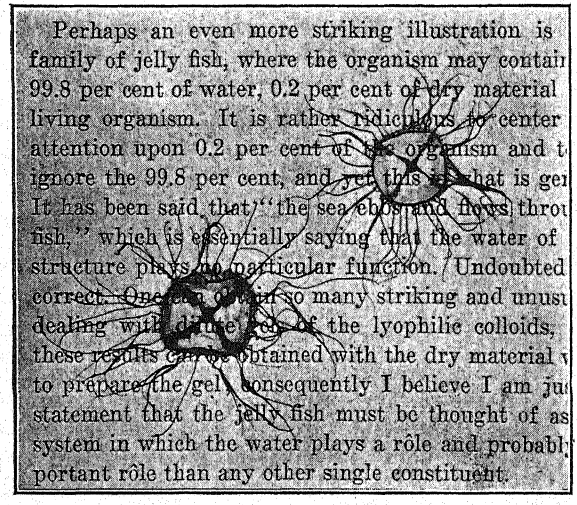


FIG. 98.—Showing adult medusae (*Gonionemus* sp.) dried down upon a magazine page. It will be noted that the amount of dry material in the umbrella is so slight that the print can easily be read through the dried organism. The dark cross bars underneath the umbrella are the radial canals to which are attached the reproductive organs, which in these specimens are filled with ripe ova.

Mammalian embryos during development show similar water relationships. Table XXXIX[37] shows the hydration capacity of pig em-

TABLE XXXIX

HYDRATION CAPACITY OF THE EMBRYONIC TISSUE OF THE PIG AT VARIOUS STAGES DURING EMBRYONIC GROWTH

Length of Embryo, mm.	Average Weight, grams	Water Content, per cent	Hydration of Tissues, per cent
2–4	.....	97.40	3750
6–7	0.313	94.07	1640
15	0.93	91.38	1062
30	2.21	91.14	1030
100	72.20	91.18	1033
160	238.57	91.71	1106
240	725.0	88.70	784

bryos at various stages during embryonic growth. In the 15-day-old embryos (2–4 mm.) there is a 3,750 per cent hydration of the tissue colloids. At the 6–7 mm. stage this has dropped to 1,640 per cent. These embryos, each containing only 0.018 gram of dry matter, are nevertheless fairly well-differentiated biological organisms and actively respond by movement to tactile stimuli. Shortly before the 15-mm. stage the hydration capacity falls to a new level of approximately 1000 per cent and remains at this new level until shortly after reaching the 160-mm. stage when it again falls to a lower level which persists until near birth. After birth a still lower level is attained.

The constituents that go to make up a living organism may be classified into five great groups, (1) proteins, (2) carbohydrates, (3) fats and lipids, (4) the inorganic salts, and (5) water. There are, to be sure, a number of compounds which do not fall definitely within one or another of these groups, but the actual amounts of such compounds are extremely small.

Of these various groups, the proteins belong to the class of lyophilic colloids. They possess the power of becoming strongly hydrated, and the large amount of water present in living organisms is probably mainly held through this affinity of the proteins for water.

So far as cell protoplasm is concerned, carbohydrates can be largely

[37] Wilkerson, V. A., and Gortner, R. A., The Chemistry of Embryonic Growth. III. A Biochemical Study of the Embryonic Growth of the Pig with Special Reference to Nitrogenous Compounds, *Am. J. Physiol.*, 102: 153–166 (1932); *cf.* also Rubner, M., Die Beziehung des Kolloidalzustandes der Gewebe für den Ablauf des Wachstums, *Biochem. Z.*, 148: 187–221 (1924), who has critically discussed various factors regarding the interrelationships of water and vital elements in physiological processes and growth.

looked upon as a source of energy, the carbohydrate content of true protoplasm being relatively low. In the plant kingdom, however, the structural elements and intracellular constituents are largely of carbohydrate nature, polysaccharides in most instances. Many of these polysaccharides are highly hydrophilic and combine with relatively large amounts of water.

The fats and lipids of living organisms exist in the form of emulsions. In the study of such emulsions, attention has been chiefly directed toward the fats, relatively little attention having been devoted to the aqueous phase. Adipose tissue usually contains as much, if not more, water than it does fat, and in some instances it is possible to break down adipose tissue by limiting the intake of water or by the use of large doses of drastic diuretics. Certain of the phosphatides, such as lecithin, are in themselves extremely hydrophilic and exist in the colloidal state and react as typical lyophilic colloids.

The salts and other true solutes present in the biological organism may be, to a very considerable extent, molecularly dispersed in the water and, on the other hand, may exist in a more or less fixed condition by being adsorbed upon the surface of the biocolloids. If they are adsorbed, they must be regarded as a part of the colloid system, the reaction of the surface upon which they are adsorbed being modified accordingly.

As already indicated, the water in a biological organism may exist in part as liquid water containing the truly dissolved solutes and providing the dispersions medium for the gels and sols making up the organism. A large part of the water, however, is "bound" by the colloidal micelles, and in this bound condition may exhibit entirely different properties from water in bulk. Within the last few years more and more attention has been given to methods whereby this bound fraction of the water can be differentiated from the total moisture content. Every organ and cell of a biological organism has a definite fluid content and a turgidity which is regulated by the biocolloids present in that organ or cell. In some instances a considerable variation in water content and turgidity may take place and life still be possible; in others, rather minor changes will cause the death of a cell.

THE BOUND WATER OF THE BIOCOLLOIDS

It will be impossible in the space at our disposal to discuss adequately all the interrelationships between the phenomena of the hydration of the biocolloids and the role that water plays in living processes. A number of general reviews are available in this field[38] which may

[38] For general references the student is referred to: Gortner, R. A., The State of Water in Colloidal and Living Systems, *Trans. Faraday Soc.*, 26: 678–686 (1930) (*cf.* also discussion, pp. 686–704); Jones, I. D., and Gortner, R. A., Free and Bound Water in Elastic and Non-Elastic Gels, *J. Phys. Chem.*, 36: 387–436

be consulted, and there is every indication that additional general papers will appear at frequent intervals, inasmuch as this field of research is exceedingly active at the present time.

ORIGIN OF THE BOUND-WATER CONCEPT.—Apparently the first suggestion that water might exist in living tissues in a state different from that of water as we know it in bulk was made by Balcar, Sansum, and Woodyatt [39] in a paper concerned with water relationships and the phenomenon of fever. They suggested as a theory that there might be a bound $\rightleftharpoons$ free water equilibrium existing in the normal body tissues and that any disturbance of this equilibrium would bring about a pathological condition, such as fever. They noted, however, that at the time they proposed this theory no laboratory technics were available by which the bound $\rightleftharpoons$ free water equilibrium could be measured and that accordingly the theory could not be experimentally tested.

Independently Newton [40] reached a similar hypothesis from a physicochemical study of the saps from winter-wheat plants which were being studied in an attempt to ascertain what factors were responsible for winter hardiness. He found that plants which had "hardened off" under natural conditions of low temperature retained the vacuolar sap in the leaves under pressures as high as 400 atmospheres and concluded that, under the stimulus of cold, winter hardy wheat plants bound the water in the tissues on the surfaces of the hydrophilic colloids. He was successful in devising a quantitative method [41] whereby the bound water could be differentiated from the total water present in the system, and the quantitative results bore out the bound-water hypothesis.

(1932); Gortner, R. A., The Role of Water in the Structure and Properties of Protoplasm, *Ann. Rev. Biochem.*, 1: 21–54 (1932); Lloyd, D. Jordan, Colloidal Structure and Its Biological Significance, *Biol. Rev.*, 7: 254–273 (1932); Lloyd, D. Jordan, The Movements of Water in Living Organisms, *Biol. Rev.*, 8: 463–481 (1933); Lloyd, D. Jordan, Protein Swelling and Molecular Organisation, *J. Intern. Soc. Leather Trades' Chemists*, pp. 208–219 (April, 1933); Gortner, R. A., Water in Its Biochemical Relationships, *Ann. Rev. Biochem.*, 3: 1–22 (1934); Barnes, T. C., and Jahn, T. L., Properties of Water of Biological Interest, *Quart. Rev. Biol.*, 9: 292–341 (1934); Gortner, R. A., Chap. VIII, The Water Relationships of the Biocolloids in "Selected Topics in Colloid Chemistry," Cornell University Press (1937).

[39] Balcar, J. O., Sansum, W. D., and Woodyatt, R. T., Fever and the Water Reserve of the Body, *Arch. Internal Med.*, 24: 116–128 (1919).

[40] Newton, R., A Comparative Study of Winter Wheat Varieties with Especial Reference to Winter Killing, *J. Agr. Sci.*, 12: 1–19 (1922); Colloidal Properties of Winter Wheat Plants in Relation to Frost Resistance, *J. Agr. Sci.*, 14: 178–191 (1924); The Nature and Practical Measurement of Frost Resistance in Winter Wheat, University of Alberta, College of Agriculture, Research Bull. 1, 53 pp. (1923). *Cf.* also Martin, J. H., Comparative Studies of Winter Hardiness in Wheat, *J. Agr. Research*, 35: 493–535 (1927).

[41] Newton, R., and Gortner, R. A., A Method for Estimating Hydrophilic Colloid Content of Expressed Plant Tissue Fluids, *Botan. Gaz.*, 74: 442–446 (1922).

Again independently Rubner[42, 43] and his co-workers arrived at essentially the same viewpoint with respect to the ability of protoplasmic colloids to bind water and remove it from the liquid state, and they developed a second technic (calorimetric method, *vide infra*) for the estimation of the bound water.

METHODS FOR THE MEASUREMENT OF BOUND WATER.—The bound-water theory, as a factor in vital processes, was developed independently in 1919 and 1920, and at that time no methods were available for the testing of the theory. Since those dates thirteen independent methods have been developed and used. Twelve of them indicate that very appreciable quantities of bound water are associated with the biocolloids. The thirteenth method, though indicating the presence of appreciable amounts of bound water, does not yield the larger values found by the other methods. As will be indicated later, when the individual methods are discussed, there are theoretical reasons for the failure of this method to yield comparable data.

(1) *The Cryoscopic Method.*—This was the first method to be adapted to the determination of bound water in relation to physiological processes. Acting on the hypothesis that the water which was intimately associated with the hydrophilic colloids might not act as a solvent for dissolved solutes, Newton and Gortner (*loc. cit.*) added a known quantity of sucrose to a sample of plant sap containing an exactly known amount of water and determined the freezing-point depression of the plant sap both before and after the addition of the sucrose. The amount of sucrose added was just sufficient to make a molar solution of the sugar in the water which was present in the sample of sap. The initial freezing-point depression of the plant sap (Δ) and the freezing-point depression of the sap-sucrose solution (Δ_a) being known, if all the water were free to dissolve the sucrose, the quantity ($\Delta_a - \Delta$) should equal the freezing-point depression of a molar solution of sucrose. If, however, the quantity ($\Delta_a - \Delta$) should prove to be greater than the freezing-point depression of a molar solution of sucrose, it would be apparent that not all the water present in the plant sap was available to act as a solvent, and from the "excess depression of the freezing point" it would be possible to calculate the quantity of water which was unavailable as a solvent. Inasmuch as sucrose had been reported[44] to form a hexahydrate in solution, the

[42] Rubner, M., Über die Wasserbindung in Kolloiden mit besonderer Berücksichtigung des quergestreiften Muskels, *Abh. preuss. Akad. Wiss., Phys.-math. Klasse*, pp. 3–70 (1922).

[43] Thoenes, F., Untersuchungen zur Frage der Wasserverbindung in Kolloiden und tierischen Geweben, *Biochem. Z.*, 157: 174–186 (1925); Physikalisch-chemische Untersuchungen zur Wasserbindung tierischer Gewebe, *Monatschr. Kinderheilk.*, 29: 378–383 (1925).

[44] Scatchard, G., The Hydration of Sucrose in Water Solution as Calculated from Vapor-Pressure Measurements, *J. Am. Chem. Soc.*, 43: 2406–2418 (1921).

molar depression (K_m) of sucrose was taken as 2.085° C., instead of the usual 1.86° C. The equation proposed for the determination of bound water was

$$\frac{\Delta_a - (\Delta + K_m)}{\Delta_a - \Delta} \times 892 = \begin{matrix}\text{grams bound water per liter} \\ \text{water in system}\end{matrix} \qquad (130)$$

where Δ_a = the freezing-point depression after the addition of a quantity of solute sufficient to form a molar solution in the amount of water present;

Δ = the depression of the freezing point of the liquid prior to the addition of the sucrose;

K_m = a molar constant for the depression of the freezing point

Grollman [45] criticized this equation, pointing out that the difference between the two freezing-point depressions would not be an accurate measure of bound water, provided that dissolved solutes were present in the original system, and modified equation (130) to take this factor into consideration. The modified equation is

$$\frac{\Delta_a - \left(\frac{1{,}000}{892}\Delta + K_m\right)}{\Delta_a - \frac{1{,}000}{892}\Delta} \times 892 = \begin{matrix}\text{grams bound water} \\ \text{per liter water in} \\ \text{system}\end{matrix} \qquad (131)$$

Equations (130) and (131) yield essentially the same values for bound water, provided that true solutes are absent from the lyophilic colloid sol. Table XL shows representative data calculated by both equations (130) and (131). It will be noted that certain of the bound-water values in Table XL are negative in sign, and one of Grollman's arguments against the bound-water hypothesis was that negative bound-water values were an impossibility. This argument is fallacious,[46] inasmuch as negative bound-water values may be expected under certain conditions. The assumptions which are made in the cryoscopic technics are (*a*) that the bound water does not dissolve the added sucrose, and (*b*) that the bound $\rightleftharpoons$ free water equilibrium is not shifted by the addition of sucrose to the system. Probably neither one of these assumptions is strictly correct. If both solute and solvent are adsorbed equally by the hydrophilic colloid, no excess depression of the freezing point will be observed and no bound water will be indicated. If the hydrophilic colloid adsorbs water in preference to the solute, there will be a positive bound-water value. If the lyophilic

[45] Grollman, A., The Vapour Pressure of Aqueous Solutions with Special Reference to the Problem of the State of Water in Biological Fluids, *J. Gen. Physiol.*, 14: 661–683 (1931).

[46] Gortner, R. A., and Gortner, W. A., The Cryoscopic Method for the Determination of "Bound Water," *J. Gen. Physiol.*, 17: 327–339 (1934).

TABLE XL

BOUND WATER IN CERTAIN PLANT SAPS AND IN GUM ACACIA SOLS
(CRYOSCOPIC TECHNIC)

Materials Used		Calculations by Equation (130)	Calculations by Equation (131)
		Bound Water, per cent	Bound Water, per cent
	Leaves of *Triticum vulgare*		
Feb. 3–18, 1922. Collected from the open (hardened)	var. Minhardi	14.4	7.49
	" Buffum	13.0	5.49
	" Turkey	9.7	4.10
	" Kanred	8.1	3.65
	" Super	4.4	−0.89
	" Fulcaster	4.3	−1.60
From greenhouse. Feb. 10–16, 1922 (non-hardened)	var. Minhardi	2.2	−3.83
	" Super	0.9	−4.37
	Cactus (stems)	8.3	5.80
	Gum acacia sols		
	1 per cent	2.37	2.32
	3 per cent	3.61	3.48
	5 per cent	4.50	4.37
	7 per cent	5.42	5.26
	10 per cent	6.39	6.15

colloid adsorbs the solute in preference to the solvent, the concentration of the solute in the free-water phase will be lowered, and the cryoscopic method will yield negative bound-water values. This is apparently what happens when electrolytes or urea are used as solutes.

Newton and Martin[47] applied the cryoscopic method with great success to a plant physiological study of drought resistance. Certain of their data are shown in Tables XLI, XLII, and XLIII.

The data in Table XLII represent the study of a transect of native vegetation growing in a habitat near Edmonton, Alberta. The species are numbered in their ecological relationships, beginning with *Bouteloua gracilis*, growing at the summit of a rather sandy hilltop, and ending with *Bechmannia erucaeformis*, growing in a slough at the bottom of the hill slope. It will be noted that there is a remarkable parallelism between the moisture relationships of the soil environment

[47] Newton, R., and Martin, W. McK., Physico-Chemical Studies on the Nature of Drought Resistance in Crop Plants, *Canadian J. Research*, 3: 336–427 (1930).

TABLE XLI

WATER BOUND BY DIFFERENT COLLOIDAL SUBSTANCES AT VARIOUS CONCENTRATIONS (CRYOSCOPIC TECHNIC. DATA OF NEWTON AND MARTIN)

Material	Actual Concentration, per cent	Bound Water per Gram Colloid, grams	Material	Actual Concentration, per cent	Bound Water per Gram Colloid, grams
Gelatin...	0.93	2.05	Dextrin.....	1.83	1.19
	1.86	1.70		3.67	0.89
	2.77	1.31		5.12	0.71
	3.66	1.17		5.54	0.66
	4.55	1.04		7.43	0.54
	5.43	0.96		9.34	0.47
				11.27	0.55
Agar.....	0.87	3.36			
	1.77	1.87	Blood fibrin..	1.91	1.61
	2.61	1.52		3.84	1.29
	3.48	1.17		5.79	0.88
	4.36	0.93		7.77	0.68
	5.24	0.82		9.78	0.56
	6.13	0.84		11.81	0.51
	7.02	0.79			
	7.92	0.79	Gum arabic..	1.79	0.38
	8.81	0.74		3.50	0.48
				5.16	0.57
Vegetable				6.75	0.56
albumin...	1.88	0.70		8.28	0.60
	3.78	0.60		9.76	0.64
	5.11	0.56		11.19	0.62
	5.70	0.56		12.56	0.61
	7.65	0.41		13.90	0.58
	9.62	0.35		15.18	0.58
				16.43	0.58

and the bound-water values as determined by the cryoscopic method. It might be assumed from the data in Table XLII that the osmotic-pressure relationships were of equal value with the water binding of the hydrophilic colloids. However, Table XLIII shows rather clearly that osmotic pressure does not measure drought resistance. In this series of wheat species the bound-water values place the wheats exactly in the order of their drought resistance as measured by field tests in many localities, the bread wheats being least and the emmers most drought resistant. Other studies [48] have shown independently that bound-water determinations measure physiological factors which are

[48] Gortner, R. A., and Rude, Rachel, Interrelationship of Certain Physico-Chemical Constants of Plant Saps, *Proc. Soc. Exptl. Biol. Med.*, 25: 630–635 (1928).

TABLE XLII

GRASSES ARRANGED IN ORDER OF DROUGHT RESISTANCE AS INDICATED BY AVERAGE BOUND WATER IN SEASONS OF 1925 AND 1926
(DATA OF NEWTON AND MARTIN)

Species	1925		1926		Average	
	Osmotic Pressure, atm.	Bound Water, per cent	Osmotic Pressure, atm.	Bound Water, per cent	Osmotic Pressure, atm.	Bound Water, per cent
1. *Bouteloua gracilis*, blue grama	34.9	16.73	59.9	28.59	47.4	22.4
2. *Stipa comata*, western spear	23.2	15.14	27.7	9.58	25.4	12.4
3. *Agropyron cristatum*, crested wheat	20.3	11.70	.....	.....	.....	.....
4. *Agropyron tenerum*, western rye	18.7	10.49	16.1	10.18	17.4	10.3
5. *Bromus inermis*, awnless brome	19.7	10.60	18.7	9.99	19.2	10.3
6. *Agropyron smithii*, western wheat	17.4	7.09	21.1	8.27	19.2	7.7
7. *Poa pratensis*, Kentucky blue	13.4	4.66	16.4	5.92	14.9	5.3
8. *Calamovilfa longifolia*, sand grass	13.2	6.91	17.8	3.32	15.5	5.1
9. *Fluminea festucacea*, prickle fescue	13.4	4.29	19.1	5.85	16.2	5.1
10. *Phleum pratense*, timothy	15.7	3.43	15.7	5.62	15.7	4.5
11. *Calamagrostis canadensis*, blue joint	11.9	3.71	13.4	3.22	12.6	3.5
12. *Panicularia grandis*, tall manna	9.7	4.40	15.5	2.22	12.6	3.3
13. *Bechmannia erucaeformis*, slough grass	11.3	3.09	12.2	1.48	11.7	2.3
Coefficients of correlation						

	n	r
Osmotic pressure, 1925, and osmotic pressure, 1926	12	0.92 ± 0.03
Bound water, 1925, and bound water, 1926	12	0.82 ± 0.06

not correlated with the usual physicochemical measurements made on plant saps. The application of the cryoscopic method to a variety of plant physiological problems [49, 50] has yielded data of great value.

[49] Greathouse, G. A., Effects of the Physical Environment on the Physico-Chemical Properties of Plant Saps, and the Relation of These Properties to Leaf Temperature, *Plant Physiol.*, 7: 349–390 (1932).

[50] Crist, J. W., Effect of Nutrient Conditions on Colloidal Properties of Cer-

TABLE XLIII

WHEAT SPECIES AND VARIETIES ARRANGED IN ORDER OF DROUGHT RESISTANCE AS INDICATED BY AVERAGE BOUND WATER IN SEASONS OF 1925 AND 1926 (DATA OF NEWTON AND MARTIN)

Species	1925		1926		Average	
	Osmotic Pressure, atm.	Bound Water, per cent	Osmotic Pressure, atm.	Bound Water, per cent	Osmotic Pressure, atm.	Bound Water, per cent
1. *Triticum dicoccum* var. common emmer.....	14.3	7.10	11.6	7.39	12.9	7.2
2. *T. durum* var. Kubanka	13.9	6.73	13.8	7.23	13.8	7.0
3. *T. turgidum* var. Alaska	13.3	6.38	13.6	7.43	13.4	6.9
4. *T. vulgare* var. Caesium	13.9	6.67	14.5	6.43	14.2	6.5
5. *T. compactum* var. hybrid 143.....	12.8	5.53	13.3	5.98	13.0	5.8
6. *T. spelta* var. white spelt	14.2	5.72	15.3	5.58	14.7	5.6
7. *T. monococcum*, einkorn	12.7	5.42	12.6	5.84	12.6	5.6
8. *T. polonicum* var. white Polish.....	13.0	4.20	11.8	4.66	12.4	4.4
9. *T. vulgare* var. Marquis	14.6	3.87	12.5	4.30	13.5	4.1
Coefficients of correlation						

	n	*r*
Osmotic pressure, 1925, and osmotic pressure, 1926	9	0.20 ± 0.22
Bound water, 1925, and bound water, 1926	9	0.96 ± 0.02

Kruyt and Winkler [51] studied the water relationships in starch sols by both viscometric methods and the cryoscopic method. They reached the conclusion that the cryoscopic method measures the closely bound water in an oriented shell of water dipoles surrounding the starch micelles, whereas viscosity methods measure not only this closely bound water but also water which is held in more diffuse layers. They find approximately 0.80 gram bound water per gram starch by the cryoscopic method, whereas the volume of the gelatinized starch micelles has apparently increased about 25 times as measured by viscometric technic (*cf.* Table VI).

(2) *The Calorimetric Method.*—Rubner's method depends upon the latent heat of fusion of ice. Whenever 1 gram of water in the form of ice changes to liquid water at 0° C., there is an absorption of

tain Vegetable Crops, Tech. Bull. 74, Michigan Agricultural Experiment Station, 27 pp. (1926).

[51] Kruyt, H. R., and Winkler, K. C., Über den Einfluss hydratierter Kolloide auf die Gefrierpunktserniedrigung, *Z. anorg. algem. Chem.*, 188: 200–204 (1930).

80 calories. In the calorimetric method the tissue under investigation is cooled to −20 or −30° C. and held at that temperature until all the "free water" is frozen. The subsequent measurement of the heat required to bring the sample to some definite temperature above 0° C. permits the calculation of the amount of water which had been converted into ice at the low temperature.

Robinson [52] details the experimental technic of the method and proposes equation (132) which is essentially identical with that which had been earlier proposed by Thoenes (*loc. cit.*):

$$X = \frac{FN(T_2 - T_3) - (WSR + W_1S_1R)}{80 - \frac{T_1}{2}} \qquad (132)$$

where F = correction for thermal capacity of calorimeter;
N = volume of water in cubic centimeters used in calorimeter;
T_2 = initial temperature of water in calorimeter;
T_3 = final temperature of water in calorimeter;
W = total weight of material;
S = specific heat of material;
R = range in temperature between T_1 and T_3;
W_1 = weight of tinfoil container in which material (W) is placed;
S_1 = specific heat of tinfoil which is 0.05;
T_1 = initial temperature of material in freezing cabinet.

Table XLIV shows the bound water in certain animal and plant tissues as well as in gelatin and agar gels, as measured by Thoenes, using the calorimetric technic. We have already indicated that in the aging of gels there is a decreased water-holding capacity. Apparently, from Thoenes' measurements of bound water in dog muscle of various ages, living gels show a similar phenomenon.

Robinson's studies [53] dealt almost wholly with the problem of winter hardiness in insects, and he found that those insect forms which were able to withstand excessively low temperatures possessed a hardening-off mechanism on being subjected to low temperature and the ability to bind relatively large amounts of the body water. Thus, the physiology of his insects parallels the phenomena which were earlier

[52] Robinson, W., Free and Bound Water Determinations by the Heat of Fusion of Ice Method, *J. Biol. Chem.*, 92: 699–709 (1931).

[53] Robinson, W., Water Binding Capacity of Colloids, a Definite Factor in Winter Hardiness of Insects, *J. Econ. Entomol.*, 20: 80–88 (1927); Relation of Hydrophilic Colloids to Winter Hardiness of Insects, Colloid Symposium Monograph, Vol. V, pp. 199–218 (1928); Response and Adaptation of Insects to External Stimuli, *Ann. Entomol. Soc. Am.*, 21: 407–417 (1928); Water Conservation in Insects, *J. Econ. Entomol.*, 21: 897–902 (1928); and A Study of the Effect of Surgical Shock on Insects, *J. Agr. Research*, 37: 743–748 (1928).

TABLE XLIV

PERCENTAGES OF BOUND AND FREE WATER IN CERTAIN ANIMAL AND PLANT TISSUES AS WELL AS OF GELATIN AND AGAR GELS (DATA OF THOENES)

Material	Age	*p*H	Total Water, per cent	Free Water, per cent	Bound Water, per cent	Bound Water for Each Gram of Dry Matter, grams
Dog muscle.....	24 hours	.....	85.7	59.0	26.7	1.86
Dog muscle.....	3 weeks	.....	83.8	60.4	23.4	1.44
Dog muscle.....	4 weeks	.....	83.3	59.7	23.6	1.40
Dog muscle.....	Several months	.....	79.3	55.1	24.2	1.16
Dog muscle.....	Several months	.....	82.0	62.9	19.1	1.06
Dog muscle.....	Several months	.....	79.7	58.3	21.4	1.05
Guinea pig muscle..	Young (160 grams)	.....	81.6	61.5	20.1	1.09
Guinea pig muscle..	Old (600 grams)	.....	79.6	60.5	19.2	0.94
Laminaria.....		5.5	57.2	21.25	37.8	0.92
Laminaria.....		6.2	69.8	32.4	37.4	1.19
Laminaria.....		8.0	62.5	28.3	34.2	0.91
Gelatin.....		5.3	87.0	62.8	24.2	1.86
Gelatin.....		4.3	86.4	60.25	26.2	1.92
Gelatin.....		3.0	87.1	59.5	27.6	2.14
Agar.....		5.5	94.1	69.55	24.55	4.15

demonstrated by Newton in winter hardy wheats. Figure 99 shows the bound and free water changes as a function of temperature and time which Robinson observed during the hardening-off period for the pupae of *Telea polyphemus*. In the granary weevil Robinson found an exactly opposite relationship to hold. The curves for both bound and free water in this insect as a function of temperature and time are shown in Fig. 100. Lowering the temperature releases bound water and increases the amount of free water. The granary weevil is not winter hardy and dies usually by the time the temperature

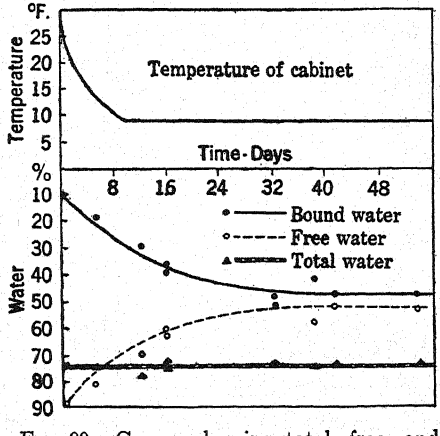


FIG. 99.—Curves showing total, free, and bound water content of the pupae of *Telea polyphemus* in relation to temperature. (Data of Robinson.)

reaches 0° C. It must be looked upon as a drought-resistant organism, living as it does in stored grain which has a moisture content of 12 per cent or less. It is necessary for such insects to conserve in some manner the water of their body fluids, and this it apparently does by utilizing more or less the same mechanism which characterizes xerophytic plants, the binding of the water on lyophilic colloids. The granary weevil is able to use metabolic water for its body processes. Lowering the temperature apparently alters the gel structure of its tissues and brings about a release of bound water.

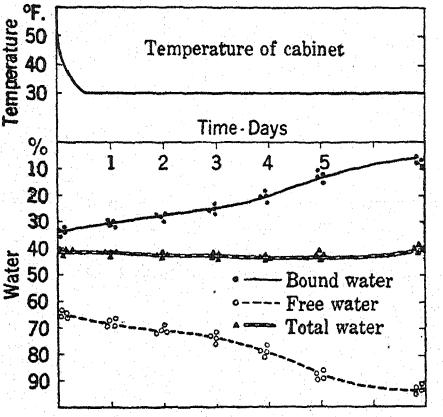


FIG. 100.—Curves showing the total, free, and bound water of the granary weevil, *Sitophilus granarius*, in relation to temperature. (Data of Robinson.)

It would appear from the observations of Robinson, Newton, and Thoenes that the cell activities of both plant and animal organisms are in a considerable degree regulated by a bound $\rightleftharpoons$ free water equilibrium which can under certain conditions of stress be shifted in one direction or the other in order to provide for the preservation of the species.

A similar shift in bound$\rightleftharpoons$free water equilibrium is reported by Kehar and McCollum [54] to occur in cardiac muscle after ventricular fibrillation. They report 18.02 per cent of bound water in normal heart muscle and 14.3 per cent after 5 minutes of fibrillation by electrical stimulation.

Helen Chrysler [55] studied the bound-free water relationships in kelp stipe at various percentages of hydration. Figure 101 shows the essential data. In Fig. 102 the data are plotted on a logarithmic scale. It will be noted that the data in Fig. 102 fall on a straight line such as one would expect from an adsorption isotherm. At 156 per cent hydration of the kelp stipe the bound water exactly equals the free water. Below this degree of hydration, the bound water exceeds the amount of free water; above it the free water exceeds the amount of bound water.

[54] Kehar, N. D., and McCollum, E. V., Bound Water in Cardiac Muscle in Relation to Ventricular Fibrillation, *Am. J. Physiol.*, 110: 485–487 (1934).

[55] Chrysler, Helen L., Amounts of Bound and Free Water in an Organic Colloid at Different Degrees of Hydration, *Plant Physiol.*, 9: 143–155 (1934).

(3) *The Dilatometric Method.*—When water is transformed into ice the volume of the solid is greater than the volume of the liquid.

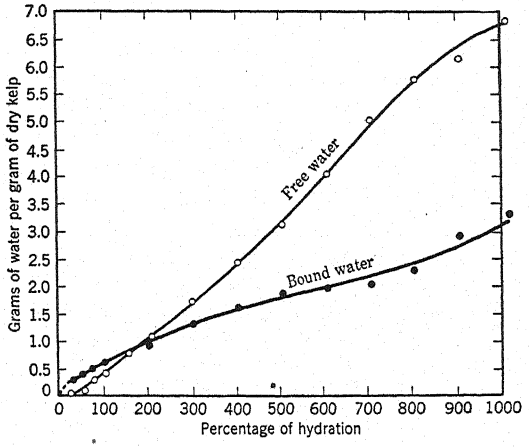


FIG. 101.—Showing the "bound" and "free" water in kelp stipe at different percentages of hydration. (Data of Chrysler.)

This expansion was used by Foote and Saxton [56] to measure the "combined water" in inorganic systems such as SiO_2—H_2O, Al_2O_3—H_2O,

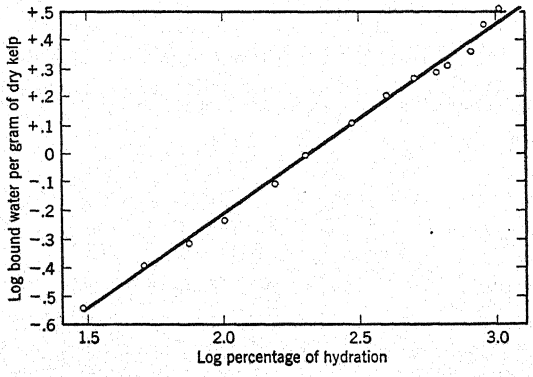


FIG. 102.—The data of Fig. 101 plotted on logarithmic scale. (Data of Chrysler.)

[56] Foote, H. W., and Saxton, B., The Effect of Freezing on Certain Inorganic Hydrogels, *J. Am. Chem. Soc.*, 38: 588–609 (1916); The Freezing of Water Absorbed in Lampblack, *J. Am. Chem. Soc.*, 39: 627–630 (1917); The Effect of Freezing on Certain Inorganic Hydrogels. II., *J. Am. Chem. Soc.*, 39: 1103–1125 (1917).

Fe_2O_3—H_2O, and carbon—H_2O. At about the same time Bouyoucos [57] used the dilatometer to determine the "unfree" water in soils, *i.e.*, the water which was not available for plant growth, and his studies of soil-plant relationships were later extended by McCool and Millar.[58] Lott [59] and Rosa [60] later used the dilatometer to study water relationships in winter hardiness. A detailed study of the dilatometric technic in its relationships to the bound-free water equilibrium was made by Jones and Gortner (*loc. cit.*), who studied not only elastic gels, such as gelatin and egg white, but certain of the non-elastic gels, such as silica gel and ferric oxide gel. Figure 103 shows a typical experiment

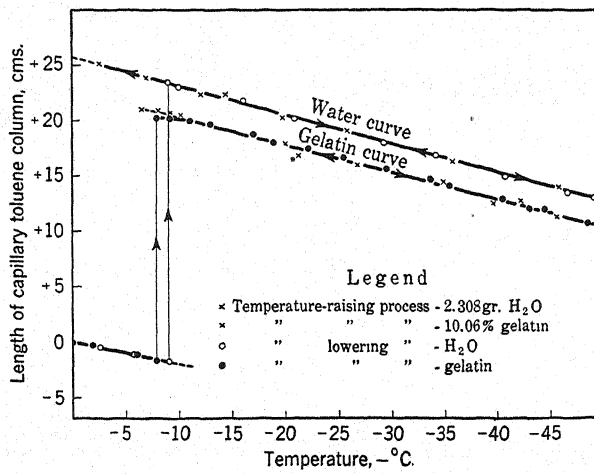


FIG. 103.—Dilatometric curves for water and water containing added gelatin, showing that a part of the water is "bound" by the gelatin. (Data of Jones and Gortner.)

in which the system gelatin-water is compared with an equal weight of water containing no hydrophilic colloids. In each instance contraction of the system proceeded to approximately −10° C. Freezing then caused an expansion of the system, and a continued lowering of the

[57] Bouyoucos, G. J., Measurement of the Inactive, or Unfree Moisture in the Soil by Means of the Dilatometer Method, *J. Agr. Research*, 8: 195–217 (1917); Classification and Measurement of the Different Forms of Water in the Soil by Means of the Dilatometer Method, *Tech. Bull.* 36, Michigan Agricultural Experiment Station (1917).

[58] McCool, M. M., and Millar, C. E., Use of the Dilatometer in Studying Soil and Plant Relationships, *Botan. Gaz.*, 70: 317–319 (1920).

[59] Lott, R. V., Correlation of Chemical Composition with Hardiness in Brambles, Missouri Agricultural Experiment Station Bull. 95, 22 pp. (1926).

[60] Rosa, T. J., Jr., Investigations on the Hardening Process in Vegetable Plants, Missouri Agricultural Experiment Station Bull. 48, 97 pp. (1921).

temperature again caused a smooth straight-line contraction down to the lowest temperature investigated, −48.6° C. The water curve and the gelatin-water curve parallel each other throughout the entire contraction range. Both curves were strictly reversible. The difference in expansion observed by the water curve and the gelatin-water curve was interpreted as being due to part of the water having been bound by the gelatin and not being transformed into an ice crystal lattice at the low temperatures.

TABLE XLV

THE BOUND WATER IN GELATIN GELS AS A FUNCTION OF GEL CONCENTRATIONS (DATA OF JONES AND GORTNER)

Gel Concentration, Per Cent	Bound Water Expressed as per Cent of Total Water in System		Water Bound per Gram Dry Gelatin	
	−10° C.	−30° C.	−10° C. grams	−30° C. grams
2	9.35	9.35	4.675	4.675
8	15.10	15.19	1.888	1.899
16	16.16	16.82	1.010	1.051
32	20.56	22.43	0.643	0.701

A study of bound water as a function of the concentration of gelatin gels, using dilatometric technic, gave the data shown in Table XLV. These data, plotted logarithmically, are shown in Fig. 104, which includes likewise the log-log curve of similar data on gelatin gels as a function of gel concentration as determined by Newton and Martin, using the cryoscopic method. It will be observed that in both instances the log-log curves are straight lines, and similarly Miss Chrysler (Fig. 102) found a straight-line relationship for bound water in kelp stipe. Thus, *the cryoscopic method, the calorimetric method, and the dilatometric method all yield bound-water data which are in harmony with the view that water binding follows a typical adsorption isotherm.*

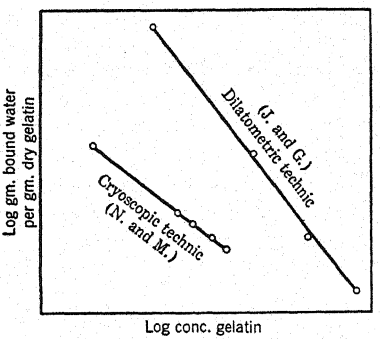


FIG. 104.—Bound water in gelatin as a function of the gel concentration. (Dilatometric data of Jones and Gortner and cryoscopic data of Newton and Martin.)

A Comparison of the Cryoscopic, Calorimetric, and Dilatometric Technics.—Sayre [61] investigated these three technics on an 18.6 per cent gum acacia sol. Table XLVI shows the comparative data. It is

TABLE XLVI

COMPARISON OF THE THREE METHODS OF MEASURING BOUND WATER IN AN 18.6 PER CENT GUM ARABIC SOLUTION (DATA OF SAYRE)

Cryoscopic Method	Calorimetric Method	Dilatometric Method
Bound Water, per cent	Bound Water, per cent	Bound Water, per cent
10.84	10.59	13.11
15.59	11.97	9.17
12.23	14.12	11.80
13.00	11.07	11.32
11.10	13.87	13.68
10.92	15.80	13.59
Mean = 12.28	Mean = 12.90	Mean = 12.11
σ = 1.83	σ = 2.02	σ = 1.74
$PE_m = \pm$ 0.50	$PE_m = \pm$ 0.56	$PE_m = \pm$ 0.48

evident that the three independent methods are measuring the same physical property and that at least on this particular system the methods could be used interchangeably. Sayre adds that, because of ease of manipulation, he personally prefers the calorimetric technic.

Similarly, St. John,[62] using the calorimetric technic, found the thick portion of egg white to contain 1.97 grams of bound water per gram of dry material. Jones and Gortner, using similar material and the dilatometric technic, found 1.55 grams of bound water.

(4) *The Osmotic-pressure Method.*—Levitt and Scarth [63] adapted the plasmolytic method (*vide infra*) to the bound-water problem for measuring the osmotic pressure of individual plant cells. They studied frost resistance in living cells as related to osmotic-pressure behavior of the cell contents and measured bound water by the modified van't Hoff equation:

$$P(V - X) = K \tag{133}$$

or

$$X = \frac{P_1V_1 - P_0V_0}{P_1P_0} \tag{134}$$

[61] Sayre, J. D., Methods of Determining Bound Water in Plant Tissue, *J. Agr. Research*, 44: 669–688 (1932).

[62] St. John, J. L., The Temperature at which Unbound Water Is Completely Frozen in a Biocolloid, *J. Am. Chem. Soc.*, 53: 4014–4019 (1931).

[63] Levitt, J., and Scarth, G. W., Frost-Hardening Studies with Living Cells, I and II, *Canadian J. Research*, 14: 267–305 (1936).

where X = the space occupied by material which is not osmotically active. If X is solid material and contributes essentially nothing to the osmotic pressure of the system, X will not vary with P. If, however, X does vary with osmotic pressure, at least a part of X can be regarded as bound water. In the hardened vacuoles of *Catalpa hybrida*, X varied from 24.5 per cent of the cell volume when the cell was in contact with 1.0 molar dextrose solution to 21.2 per cent in 3.0 molar dextrose solution. In the unhardened tissue, X ranged from 16.6 to 14.5 per cent of the cell volume. *Liriodendron* cells show similar changes. The authors conclude that by this method bound water is demonstrated *in living tissue,* but they add that it appears to be a factor of the vacuole sap rather than of the cell protoplasm.

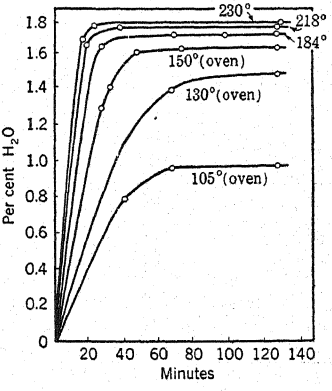


FIG. 105.—Time *vs.* moisture-loss curves for a sample of a biochemical product as influenced by the drying temperature. (Data of Nelson and Hulett.)

(5) *Drying Methods.*—We have already indicated that water is held with great tenacity upon surfaces, so that appreciable amounts of water may still remain in a colloid gel which has been heated to a rather high temperature. The experiments of Nelson and Hulett (*loc. cit.*) with reference to the probable true moisture content of various biochemical products have already been referred to. Figure 105 shows the forms of the drying curves which they obtained for various temperature and time intervals, and Table XLVII shows the moisture content which they determined by what is essentially the "official" method and the "probable true moisture content" of a variety of materials. The latter values are probably still too low, inasmuch as the authors assumed that water could not exist on surfaces above the critical temperature of water (365° C.), and as we have already seen, water can exist on surfaces at much higher temperatures. Rimington[64] is in agreement with the argument that from 2 to 7 per cent of water still adheres to protein after the protein has been dried to constant weight preliminary to carrying out an elementary analysis. He states that the values for hydrogen and oxygen in proteins are too high to agree with what we know of their amino acid composition.

(6) *The Direct Pressure Method.*—Under imbibition pressure we discussed the experiments of Reinke and the swelling of biocolloids against pressure. In the early experiments of Newton on winter hardi-

[64] Rimington, C., *Trans. Faraday Soc.*, 26: 699–702 (1930).

TABLE XLVII

DIFFERENCE BETWEEN THE MOISTURE CONTENT OF VARIOUS BIOLOGICAL PRODUCTS AS OBTAINED BY THE "OFFICIAL" METHOD AND THE PROBABLE TRUE MOISTURE CONTENT

(DATA OF NELSON AND HULETT)

	Apparent Water Content at 100° *in vacuo*, per cent	Probable True Water Content, per cent	Difference Due to Water Films Having No Appreciable Vapor Pressure at 100° *in vacuo*, per cent
Wheat flour	10.80	11.80	1.00
Cornmeal	11.34	12.25	0.91
Cornstarch	11.80	12.40	0.60
Cellulose (Swedish filter paper)	2.63	2.80	0.17
Cellulose (absorbent cotton)	5.49	5.90	0.41
Protein (edestin)	10.40	12.30	1.90

ness, the sap was expressed from the wheat leaves in a hydraulic press. Figure 106 shows certain of Newton's data. The order of the wheats is exactly the same in this figure as the bound-water values for the

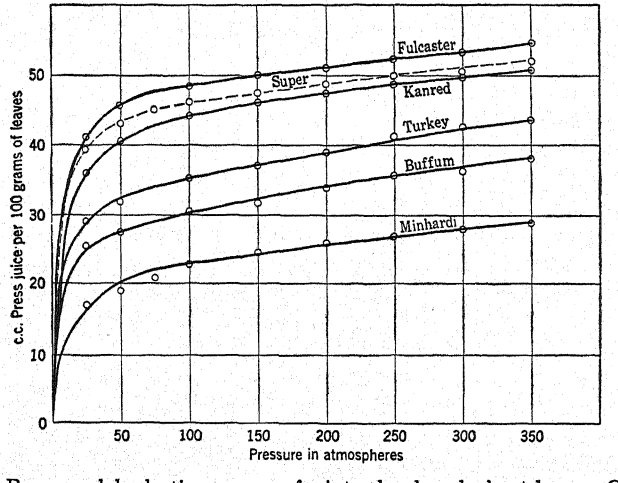


FIG. 106.—Pressure dehydration curves of winter-hardened wheat leaves. Collection made from Minnesota Agricultural Experiment Station field plots, January 21, 1922. (Data of Newton.)

saps shown in Table XL. Perhaps the most conclusive experiment is that carried out by Lloyd and Moran.[65] They constructed a hydraulic press which would maintain a constant pressure for a long period of time. Gelatin gels of various concentrations were wrapped in canvas impregnated with collodion (an ultrafilter) and subjected to pressure as long as fluid could be expressed from the gel. They found the equilibrium moisture contents of the various gels to be identical irrespective of the original concentration of the gel. At approximately a 66 per cent gel they observed that there was a relatively sharp break in the pressure-gel concentration curve and that this point was reached by a pressure approximating 8,000 pounds per square inch. Over the pressure range between 8,000 and 38,000 pounds per square inch only an insignificant increment of additional water was removed, but when the pressure was increased above 38,000 pounds per square inch, some slight amount of additional water could be squeezed out. Figure 107 shows the water content of their gelatin gels under various equilibrium pressures. They then calculated the activity of the water molecules in the gels at equilibrium with the various pressures and the temperature at which water having that activity would be expected to freeze. Table XLVIII shows certain of their experimental data and their calculations. Inacmuch as they conclude that the water in gelatin gels ranging from 68 to 76 per cent concentration has activities such that it will remain unfrozen at temperatures ranging from −73 to −158° C., it is perhaps not surprising that measurements of bound water by both the dilatometric and the calorimetric technics have indicated that appreciable quantities of water remain unfrozen in colloid gels in the temperature range of −20 to −30° C.

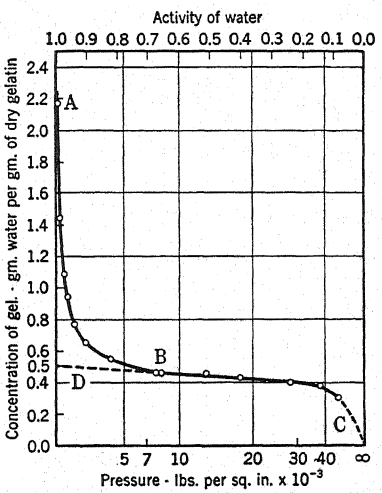


FIG. 107.—Water content and "activity" of water in gelatin gels as a function of pressure. (Data of Lloyd and Moran.)

(7) *The Specific-heat Method.*—The specific-heat method was introduced by Hampton and Mennie,[66] who note that a portion of the

[65] Lloyd, D. J., and Moran, T., Pressure and the Water Relations of Proteins. I. Isoelectric Gelatin Gels, *Proc. Roy. Soc. (London)*, 147A: 382–395 (1934).

[66] Hampton, W. F., and Mennie, J. H., Heat Capacity Measurements on Gelatin Gels, I and II, *Canadian J. Research*, 7: 187–197 (1932); *ibid.*, 10: 452–462 (1934).

TABLE XLVIII

DATA OF LLOYD AND MORAN FOR THE ACTIVITY OF WATER, GEL CONCENTRATION, GRAMS RESIDUAL WATER PER GRAM DRY GELATIN, AND THE CALCULATED TEMPERATURE AT WHICH THAT AMOUNT OF WATER WOULD FREEZE IN GELATIN GELS WHICH HAD BEEN HELD UNDER VARIOUS HYDRAULIC PRESSURES

Pressure (observed), lb. sq. in.	Activity of Water	Concentration of Gel (observed)		Calculated Equivalent Freezing Temperature, °C.	Corrected Freezing Temperature, °C.
		Gelatin, per cent	Grams Water, per Gram Gelatin		
8,300	0.658	68.3	0.46	− 43	− 73
13,300	0.513	68.3	0.46	− 67	− 94
18,300	0.398	69.9	0.43	− 94	−109
28,300	0.242	71.5	0.40	−135	−130
38,300	0.146	72.6	0.38	−211	−146
48,300	0.088	75.8	0.32	−223	−158

water in a colloid gel has a specific heat which is less than unity and this portion of the water they regard as "bound." They calculate the bound water from the equation:

$$x = \frac{aH_g + (1 - a)H_i - H_{\text{obs.}}}{H_i - H_x} \qquad (135)$$

where a = the weight of dry gelatin;
x = grams bound water per gram of gel;
H_g, H_x, and H_i = the heat capacity per gram of dry gelatin, bound water, and ice respectively;
$H_{\text{obs.}}$ = the measured heat capacity of the gel.

In a 24 per cent gelatin gel at temperatures ranging from −3 to −78.5° C., they find amounts of bound water which vary from 0.69 to 0.24 gram water per gram dry gelatin, the amount progressively decreasing as the temperature is lowered. At temperatures between −60 and −78.5°, approximately 25 per cent of the water in the 24 per cent gel is "bound," and since they found that the specific heat of the water in a gelatin gel changes with both the gel concentration and the temperature, they note that equation (132) will probably have to be modified to correct for variable specific heat factors. Hampton and Mennie find a specific heat of 0.80 for bound water. Kotukov [67] found the specific heat of bound water in gum acacia sols to range from 0.678 in a 20 per cent sol to 0.771 in a 4 per cent sol.

[67] Kotukov, A., The Heat Capacity of Bound Water (in Russian, translated title), *"Colloid J."* (U.S.S.R.), 2: 291–296 (1936).

(8) *The Refractometric Method.*—Koets,[68] working in Kruyt's laboratory, investigated the differential adsorption of water and alcohol by silica gel, using a liquid interferometer to measure change in concentration of the equilibrium solution. At low alcohol concentrations there was a slight positive adsorption of the alcohol. When the alcohol concentration was about 6 mole per cent, the alcohol and water were adsorbed in the same ratio as in the original solution. At higher alcohol concentrations there was a preferential adsorption of water, reaching a maximum at about 95 per cent alcohol. Silica gel will therefore bind water against the dehydrating forces of 95 per cent alcohol. Dumanski [69] later used the refractometer to measure bound water in various colloid systems, and in his laboratories [70] the method has been applied to such problems as bound water of wheat glutens as influenced by hydrogen-ion concentration, bound water in cheese as a function of the ripening process, bound water in bread doughs during various stages of fermentation, etc. Because of the rapidity and exactness with which measurements can be made, the refractometric technic promises to be very useful.

(9) *The Polarimetric Method.*—Koets (*loc. cit.*) also used the polariscope to study the selective adsorption of water from solutions of sucrose by silica gel. He found that equilibrium in each instance was reached at about 0.30 gram water per gram dry silica gel, irrespective of the original water content which the gel may have had. He interprets this to mean that there is a layer of oriented water molecules on the surface of the silica gel and that this layer is so rigid that sugar molecules cannot penetrate into it. Over the range of sucrose concentrations which he studied the water adsorption by the silica gel was independent of sucrose concentration.

The limitation of the polarimetric method is that it can be used only in systems where the solute is optically active and where the hydrophilic colloid can be removed prior to making the reading or where the hydrophilic colloid itself does not contribute to the optical activity of the sol. Since most biocolloids contain asymmetric carbon atoms, it is improbable that the polarimetric method will find appreciable application in biochemical studies of bound water.

(10) *The Dielectric-constant Method.*—Marinesco [71] studied various sols and gels at various concentrations with respect to changes in the dielectric constant, and from his studies concluded that appreciable quantities of the water in such systems possess a dielectric constant

[68] Koets, P., Water adsorption on silica gel, *Proc. Acad. Sci. Amsterdam,* 34: 420–426 (1931).

[69] Dumanski, A., Die Bestimmung der Menge des gebundenen Wassers in dispersen Systemen, *Kolloid Z.*, 65: 178–184 (1933).

[70] Numerous papers in Vols. I and II of *"Colloid J."* (in Russian) (1936).

[71] Marinesco, N., Polarisation diélectrique et structure des colloides, *J. chim. phys.*, 28: 51–91 (1931).

much lower than the value of 80 which characterizes water in bulk. He accordingly suggests that hydrophilic colloids are encased in a layer of oriented water molecules and that the adsorption pressure exerted on these water dipoles is so great as to immobilize the water molecules so that they behave as a rigid system. For such immobilized water molecules he finds a dielectric constant of approximately 2 and concludes that this is analogous to Bridgman's [72] ice VI which is stable above 0° in equilibrium with ice V and liquid water when the pressure equals or exceeds 6,380 kg. per sq. cm. The pressure data of Lloyd and Moran (*loc. cit.*) are in agreement with Marinesco's view, as are also other molecular orientation studies which indicate that water molecules may be adsorbed and oriented at interfaces and when so adsorbed and oriented become to all intents and purposes a part of the solid phase.

The dielectric constant method has been adapted to the measurement of the moisture content of cereal grains [73, 74] and similar agricultural products.

(11) *The X-ray Method.*—The lines of the X-ray pattern of starch and cellulose have been interpreted by Kolkmeijer, Favejee, and Heyn [75] to mean that a part of the lines belong to the crystal structure of "ice." Thus, in the starch X-ray pattern 15 of the 18 ice lines occur. Only 6 lines of the starch pattern are really different from the ice lines, and these 6 lines probably are due to the starch structure. Similarly in cellulose all the ice lines are present, although somewhat distorted by the "strong electric field of the micelle giving rise to stresses." These lines are regarded as being due to the "structure of a hydration film of a more or less deformed ordinary ice." Inasmuch as the ice crystal lattice is produced by a specific orientation of molecules, it seems logical to believe that an oriented water shell would produce an X-ray pattern such as has been observed, especially since the oriented polymolecular films of stearic acid formed by the Langmuir-Blodgett technic do show a typical X-ray diffraction pattern.[76]

Barnes and Hampton [77] attacked the bound-water problem with

[72] Bridgman, P. W., Water, in the Liquid and Five Solid Forms, under Pressure, *Proc. Am. Acad. Arts Sci.*, 47: 439–558 (1912).

[73] Burton, E. F., and Pitt, A., A New Method for the Rapid Estimation of Moisture in Wheat, *Canadian J. Research*, 1: 155–162 (1929).

[74] Berliner, E., and Rüter, R., Über dielektrische Messungen als Schnellwasserbestimmung, *Kolloid Z.*, 47: 251–257 (1929).

[75] Kolkmeijer, N. H., and Favejee, J. C. L., Structure of Emulsoid Sol Particles and Their Hydration Film, *Nature*, 132: 602–603 (1933); Kolkmeijer, N. H., and Heyn, A. N. J., The Hydration Film of Cellulose in Cell-Walls, *Proc. Acad. Sci. Amsterdam*, 37: 92–93 (1934).

[76] Germer, L. H., and Storks, K. H., The Structure of Langmuir-Blodgett Films of Stearic Acid, *Proc. Natl. Acad. Sci. U. S.*, 23: 390–397 (1937).

[77] Barnes, W. H., and Hampton, W. F., An X-ray Method for the Study of "Bound Water" in Hydrophilic Colloids at Low Temperatures, *Canadian J. Research*, B13: 218–227 (1935).

X-ray technic but adopted somewhat different criteria in interpreting their results. Using gelatin gels of various water contents they froze the gels rapidly and photographed the X-ray diffraction pattern in these frozen gels over a series of temperature ranges. They conclude that all the water is bound in a gelatin gel when certain characteristic ice bands do not appear in the X-ray diffraction pattern, and on this basis find that "the amount of bound water in gelatin gels is independent of temperature, at least between about −25° C. and −50° C. and corresponds to about 0.44 gm. of water per gm. of dry gelatin."

(12) *The Heat-of-wetting Method.*—We have already noted that the solvation of lyophilic colloids is usually accompanied by a positive heat of adsorption, and in Fig. 78 have shown the heat of adsorption of silica gel with variation in water content. Table XLIX shows the

TABLE XLIX

CALCULATIONS OF THE "HEAT LOSS PER GRAM OF WATER" BY ADSORPTION OF WATER ON SiO_2 AND GELATIN

Silica[78]			Gelatin[79]		
(A) Water per Gram SiO_2, grams	(B) Heat of Wetting per Gram SiO_2, calories	B/A Heat Evolved per Gram H_2O, calories	(A) Water per Gram Gelatin, grams	(B) Heat of Wetting per Gram Gelatin, calories	B/A Heat Evolved per Gram H_2O calories
0.0238	7.71	324	0.0053	2.1	396
0.0535	13.67	256	0.0168	5.3	316
0.0859	16.83	196	0.0315	7.0	222
0.1292	18.39	142	0.0406	8.9	219
0.1883	19.50	103	0.0514	10.0	194
0.2736	20.75	75.8	0.0738	13.1	177
0.3995	22.30	55.8	0.1030	18.4	177
0.4635	23.06	49.7	0.1070	19.1	178
0.5648	24.34	43.0	0.1328	22.2	168
0.6478	25.10	38.7	0.1932	30.6	158
0.7694	25.81	33.5	0.2420	33.2	137

heat of adsorption of water on silica and gelatin recalculated in terms of "heat evolved per gram water."

At the lower water contents the heat of wetting liberates 300–400 calories per gram water adsorbed. When liquid water is transformed to the ice crystal lattice, the heat of crystallization is only 80 calories per gram. If we assume that the heat of wetting comes largely from

[78] Columns (A) and (B) from Nutting (*loc. cit.*)
[79] Columns (A) and (B) from Katz, J. R., *Kolloidchem. Beihefte*, 9: 1–182 (1917).

water molecules which lose a part of their kinetic energy when they are oriented upon an interface, it is self-evident that those water molecules which have lost more than 80 calories per gram cannot rearrange themselves at a temperature below 0° C. into an ice crystal lattice which is characterized by a latent heat of fusion of 80 calories per gram. In the case of the silica noted in Table XLIX, the limiting value of 80 calories per gram is reached in a gel containing 24 per cent of water. Jones and Gortner (*loc. cit.*), using the dilatometric method, found 33.2 per cent of bound water in a silica gel system. The agreement here is at least qualitative. In the gelatin series the data in Table XLIX indicate that, even in a gel containing 0.24 gram water per gram dry gelatin, the limit of 80 calories per gram has not yet been reached. Extrapolation of the curve for the B/A figures for gelatin indicates that the limiting value will be reached somewhere in the neighborhood of 0.5 gram bound water per gram dry gelatin, and this is essentially the value indicated by Lloyd and Moran's pressure experiments, as well as by independent data which have been secured by the cryoscopic, calorimetric, and dilatometric methods.

(13) *The Vapor-pressure Method.*—The only method which so far has failed to indicate that relatively appreciable quantities of bound water are associated with lyophilic sols and gels is the vapor-pressure method. This is the method which has been used by Hill,[80] Grollman (*loc. cit.*), and Briggs.[81] Hill finds only about 3 per cent of the water in blood that can be regarded as "bound" and obtains similar results for casein and egg white systems. It should be noted, however, that these systems contain from 70 to 85 per cent water, so that even 3 per cent of this amount would be from 7 to 17 per cent of bound water based on the dry matter content of the system. Briggs points out that Hill applied the theory of dilute solutions to the systems under investigation, whereas in reality lyophilic colloid systems appear to obey more nearly the laws of ideal concentrated solutions, and when the data are calculated from this standpoint, isoelectric casein appeared to bind about 0.50 gram water per gram dry casein. Briggs prefers to calculate the activity coefficient of water in colloidal systems at various water contents and to measure "activity depressions" in the same way that freezing-point depressions are used in the cryoscopic technic, or vapor-pressure depressions in the vapor-pressure technic. Taking bound water to mean that water which has an activity coefficient of a water molecule at −20° C., Briggs finds that this point is reached at

[80] Hill, A. V., and Kupalov, P. S., The Vapour Pressure of Muscle, *Proc. Roy. Soc.* (*London*), B, 106: 445–477 (1930); Hill, A. V., The State of Water in Muscle and Blood and the Osmotic Behaviour of Muscle, *Proc. Roy. Soc.* (*London*), B, 106: 477–505 (1930).

[81] Briggs, D. R., Water Relationships in Colloids, I. Vapor Pressure Measurements on Elastic Gels, *J. Phys. Chem.*, 35: 2914–2929 (1931); II. "Bound" Water in Colloids, *J. Phys. Chem.*, 36: 367–386 (1932).

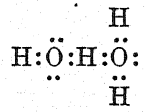
an activity coefficient of 0.8221. Using this definition and relative vapor pressure-water content curves, Briggs finds the following bound water values per gram dry matter for various materials: casein (isoelectric) 0.18 gram, gelatin 0.33 gram, gum acacia 0.32 gram, fibrin 0.33 gram, and agar 0.37 gram. These are appreciable quantities, somewhat comparable but distinctly less than values obtained by other technics. They do indicate, however, that appreciable quantities of bound water can be demonstrated by the vapor-pressure technic.

There is one fundamental reason why the vapor-pressure technic may be expected to yield minimal values of bound water and values which are lower than would be found by other technics. The assumption which underlies a relative vapor-pressure measurement is that the system is in equilibrium. *No lyophilic colloid gel is ever in strict equilibrium with the dispersions medium.* Syneresis is continually taking place. The gel is continually aging, and the dispersions medium is being continually increased at the expense of the solvation of the gel. Accordingly in the vapor-pressure measurement one measures the vapor pressure of the most dilute portion of the gel system, the vapor pressure of the liquid of syneresis, and since we have already seen that the liquid of syneresis is a much more dilute colloid system than a gel proper, it would be anticipated that vapor-pressure measurements would give distinctly lower values for bound water than those technics which measure primarily that portion of the water intimately associated with the surface of the lyophilic colloids. Lamb and Coolidge (*loc. cit.*) early recognized this behavior, for in discussing their adsorption pressure measurements they state, "If this liquid is under as great a pressure as we have computed, one might at first glance expect its vapor pressure to be considerably increased. But this pressure is not an unbalanced one; it is not applied solely to the liquid phase, but exerts its effect upon the escaping gaseous molecules as well. As a result it can have no tendency to increase the vapor pressure of the liquid phase, and indeed its net effect must be to lessen the vapor pressure of the liquid film, at least until this film extends beyond the limit of its sphere of action."

THEORETICAL CONSIDERATIONS OF THE NATURE OF BOUND WATER.—We have discussed the hydration shell of hydrophilic colloids and some of the experimental evidence that the water molecules in this hydration shell differ from the state which characterizes water molecules in ordinary liquid water. Heats of hydration, dielectric-constant measurements, and the contraction of the system, colloid-water, including the pressure studies of Lloyd and Moran, all indicate that the H_2O molecules in bound water are more closely packed and are probably specifically oriented in relationship to each other, possibly in a more or less true crystal lattice which is more densely packed than the crystal lattice of ice. We have seen from the work of Blodgett that ori-

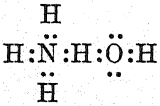
ented molecular films may be many molecular diameters in thickness. The water molecule is rather highly unsymmetrical and possesses a high dipole moment. Smyth [82] notes that "In the water molecule, the positive ends of two large doublets lie near the surface causing a very strong field of force around the molecule, so that the molecules affect one another greatly, strong association occurs, and the liquid is highly abnormal." When water comes in contact with a polar surface the electrical forces of that polar surface will cause the water dipole to be "associated" with it under the same forces that cause the association of water in bulk. If the surface attractions are more intense than the attractions between the water molecules themselves, then the intensity of the water binding at surfaces will be greatly increased and the water molecules will be held more rigidly and more closely packed than they are in liquid water.

The phenomenon of the association of liquids has been interpreted by Latimer and Rodebush [83] and by Huggins [84] on the basis of a "hydrogen bond." A hydrogen bond may be visualized as a hydrogen atom which is strongly associated with two electronegative atoms and thus acts as a "bridge" to hold these electronegative atoms relatively close together. Latimer and Rodebush state, "In terms of the Lewis theory, a free pair of electrons on one water molecule might be able to exert sufficient force on a hydrogen held by a pair of electrons on another water molecule to bind the two molecules together. Structurally this may be represented as:



Such combinations need not be limited to the formation of double or triple molecules. Indeed the liquid may be made up of large aggregates of molecules, continually breaking up and reforming under the influence of thermal agitation.

"Such an explanation amounts to saying that the hydrogen nucleus held between 2 octets constitutes a weak 'bond.' Ammonium hydroxide,



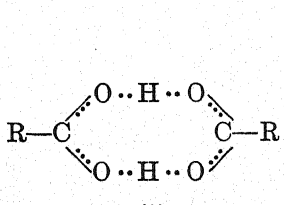
[82] Smyth, C. P., Dielectric Constant and Molecular Structure, Am. Chem. Soc. Monograph, 55: 180, Chemical Catalog Company, New York (1931).

[83] Latimer, W. M., and Rodebush, W. H., Polarity and Ionization from the Standpoint of the Lewis Theory of Valence, *J. Am. Chem. Soc.*, 42: 1419–1433 (1920).

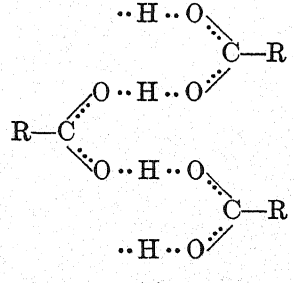
[84] Huggins, M. L., Hydrogen Bridges in Ice and Liquid Water, *J. Phys. Chem.*, 40: 723–731 (1936); Hydrogen Bridges in Organic Compounds, *J. Org. Chem.*, 1: 407–456 (1936).

is an example in which the union is fairly strong. . . . There seems to be no reason for believing that gradations may not exist all the way from the case of ammonium chloride, where the hydrogen is definitely transferred from the chlorine to the ammonia, to the case in the association of water where the hydrogen is still held quite firmly to the original water molecule."

Huggins points out that the hydrogen bond is rather readily formed between atoms of fluorine, oxygen, nitrogen, phosphorus, and sulfur, and that the strength of the bond is in the order noted. The phenomena that result are essentially the same as would result if hydrogen were considered as being not only univalent but likewise divalent. Thus, in hydrofluoric acid we must consider the undissociated molecule as having the composition H_2F_2 which gives rise to a hydrogen ion (H^+) and a hydrofluoride anion HF_2^- $\left(:\ddot{F}:H:\ddot{F}:\right)^-$. In this anion the hydrogen appears to be held extremely rigidly and essentially equidistant from the two atoms of fluorine. Huggins has calculated the dissociation energy of the FHF bridge as 6.2 kilogram calories per mole with the energy of the OHO bridge somewhat less but still very appreciable. The hydrogen bridge explains the X-ray structure of the ice crystal where each oxygen atom is surrounded by four equidistant hydrogen atoms tetrahedrally arranged, and these in turn are "bridged" to other oxygen atoms, thus building up a symmetrical structure. The hydrogen bridge likewise explains the association and the different crystal forms of the aliphatic acids. It is well known that the fatty acids even in the vapor state appear to be bimolecular. They probably exist in the following (*A*) form, although a second (*B*) form in which the hydrogen bridge permits the formation of strings of molecules is likewise probable. Oxalic acid crystals of each form are known.



(A)



(B)

When the hydrogen is essentially equidistant between the two electronegative atoms, the bridge is said to be symmetrical. If the hydro-

gen atom oscillates about a position closer to one of the electronegative atoms than to the other, the bridge is unsymmetrical and, of course, is weaker between the hydrogen atom and the electronegative atom which is at the greater distance. The strength of the bridge increases as the strength of the attraction between the hydrogen atom and the electronegative atoms is increased. We have seen that the forces of adsorption may be of relatively enormous magnitudes, *e.g.*, holding water on SiO_2 or Al_2O_3 at temperatures exceeding 500° C. This indicates that the force bonding the water on such surfaces is very intense, and the probable explanation for these forces may be found in the strength of the hydrogen bridge.

Lloyd and Moran give a somewhat similar picture for the forces holding bound water in their gelatin gels. They point out that "The hydration of proteins can occur by the formation of a co-ordinate link between a water molecule and certain groups in the protein structure for instance, the positively charged basic groups of the proteins readily accept a pair of electrons from the oxygen of a water molecule on to one (or more) of the hydrogen atoms.

"Conversely the negatively charged acidic groups of the proteins readily donate a pair of electrons from the singly bound oxygen atom to the hydrogen of a water molecule.

"Neutral groups, such as OH, NH_2, NH, COOH, can similarly form co-ordinate links with water molecules either by the donation or the acceptance of a pair of electrons. . . . It should be noticed, however, that there is no way of constructing a co-ordinate link between a protein molecule and a water molecule except by donating a pair of electrons on to a chemically combined hydrogen atom. . . .

"The experimental data . . . show that 1 gram of dry gelatin carries 0.5 gram of closely bound water. One molecule of gelatin (mol. wt. 34,500) is therefore closely bound to 960 water molecules, a figure very close to the calculated number of possible co-ordination centres."

We may conclude therefore that the forces which bind water on the surface of the lyophilic colloids are of the same nature as the forces which cause the association of water in bulk and which immobilize water molecules in the ice crystal lattice. However, there is evidence that these forces on a surface or at an interface may be of greater magnitude than the forces of association of water molecule for water molecule or the forces which tend to arrange water molecules in the ordinary ice crystal lattice. Therefore at least a part of the molecules of the bound water film may be expected to have an activity which is less than the activity of the H_2O molecule in the ordinary ice lattice.

Some Indirect Evidences of Bound Water.—Some indirect evidence supports the bound-water hypothesis. Figure 108 shows the

respiration curve of wheat [85] at various moisture contents. At about 14.75 per cent of moisture the curve breaks sharply and respiration increases nearly 200 per cent for an additional 1 per cent of moisture. It appears probable that at or close to 14.75 per cent moisture all or nearly all the water is bound in the wheat kernel and that appreciable amounts of free water are present only when the moisture content exceeds 14 per cent. Metabolic activity accordingly would appear to be related to the increase in free water, whereas dormancy would be associated with bound water.

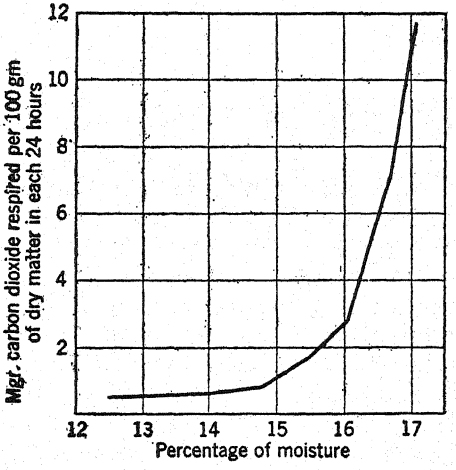


FIG. 108.—Respiration (heating) curve of wheat in relation to moisture content of grain. (Data of Bailey.)

Whitcomb and Sharp [86] studied the germination of wheat kernels which had been frozen at different stages of maturity. Kernels containing 56 per cent or more of moisture were almost uniformly killed by freezing, whereas kernels containing 50.6 per cent or less of moisture were highly resistant. A difference of 5.4 per cent of moisture in the kernel cannot account for the differences in germination, and it appears probable that a large part of the water represented by the 50.6 per cent moisture content is in a bound form.

Jackson [87] studied the degree to which earthworms (*Lumbricus terrestris*) could be desiccated without loss of vitality. The earthworms were allowed to dry down at room temperature on filter paper, the loss in weight being ascertained by weighing at frequent intervals. Before the beginning of the experiment the earthworms had been kept for a day or two barely covered with tap-water until the weight reached a constant value. The organisms which were desiccated to a point where they had lost not more than 43 per cent of their body weight invariably recovered when returned to water. On the other hand, those which had lost 50 per cent of their body weight invariably failed to revive. There

[85] Bailey, C. H., and Gurjar, A. M., Respiration of Stored Wheat, *J. Agr. Research*, 12: 685–713 (1918).

[86] Whitcomb, W. O., and Sharp, P. F., Germination of Frozen and Non-Frozen Wheat Harvested at Various Stages of Maturity, *J. Agr. Research*, 31: 1179–1188 (1925).

[87] Jackson, C. M., Storage of Water in Various Parts of the Earthworm at Different Stages of Exsiccation, *Proc. Soc. Exptl. Biol. and Med.*, 23: 500–504 (1925).

is a critical threshold below which water loss cannot go and the organism still recover, this threshold lying somewhere between 43 and 50 per cent loss of body weight. It seems altogether improbable that there would be such a sharp critical threshold if all the water in the organism were present in the same form, and these observations, taken in conjunction with those already noted, can be best interpreted in terms of a free $\rightleftharpoons$ bound water equilibrium where the free water content is exhausted at somewhere between 43 and 50 per cent of the body weight of the organism.

Coblentz [88] points out that water possesses pronounced infra-red absorption bands at 1.5 μ and 2.0 μ. He cites a single experiment with a gelatin film and notes that the general appearance of the infra-red transmission curve is entirely unlike that of water or of substances containing water, adding, "In fact, the whole curve appears as though the water was present as 'water of constitution.'" No data are given by Coblentz as to the percentage of water present in his "gelatin film."

Infra-red absorption studies promise to be a fourteenth method for the study of bound water. Such measurements have recently [89] been made on various organic compounds containing hydroxyl oxygens and nitrogen atoms in studying hydrogen bond formation, the authors pointing out that the characteristic absorption spectrum of the hydroxyl group disappears when the hydrogen bond is present. Their observations lend weight to the observation of Coblentz and to the theories which we have just discussed. Furthermore, Boswell [90] has been studying infra-red absorption spectra in lyophilic colloid systems and finds that in certain lyophilic colloids the infra-red absorption band characteristic of the hydrogen bonds appears when water is adsorbed from the vapor phase.

Imbibition of Lyophilic Colloids in Normal Physiological Processes.—Imbibition and syneresis play very important roles in the digestive and assimilative processes. In fact, osmotic phenomena within the living organism may be regarded, at least in part, as dependent upon imbibition forces.

The osmotic membranes or semi-permeable membranes of living organisms are invariably lyophilic gels. Much has been written in regard to the chemical nature of such membranes. Overton postulated

[88] Coblentz, W. F., The Role of Water in Minerals, *J. Franklin Inst.*, 172: 309–335 (1911).

[89] Hilbert, G. E., Wulf, O. R., Hendricks, S. B., and Liddel, U., The Hydrogen Bond between Oxygen Atoms in Some Organic Compounds, *J. Am. Chem. Soc.*, 58: 548–555 (1936); Hendricks, S. B., Wulf, O. R., Hilbert, G. E., and Liddel, U., Hydrogen Bond Formation between Hydroxyl Groups and Nitrogen Atoms in Some Organic Compounds, *J. Am. Chem. Soc.*, 58: 1991–1996 (1936); *cf.* also Schaefer, Cl., and Matossi, F., Das ultrarote Spektrum, Julius Springer, Berlin (1930), for technic and theory.

[90] Buswell, A. M., personal communication.

that the plasma membrane is of a lipid nature, because anesthetics and fat-soluble materials pass readily into a cell, whereas salt solutions and substances which are typically water-soluble, as contrasted with lipid-soluble, pass with difficulty.

The plasma membrane is not necessarily a structure formed by vital forces. A purely mechanistic explanation can be given for its formation. It would seem as though the formation of the plasma membrane was the inevitable consequence of the chemical composition of protoplasm. As already noted, protoplasm is composed, so far as the organic portion is concerned, largely of proteins together with a certain proportion of fats and lipids. If a protein-fat-lipid mixture is allowed to come in contact with water or a salt solution at an interface, an interfacial tension will develop, and *accordingly Gibbs' equation* (122) *will determine what compounds are present in the interface.* Those substances which decrease interfacial tension will be concentrated in the interface. Since lipids, such as lecithin, and the other phospholipids, as well as fats and salts of the fatty acids, markedly lower interfacial tension, these compounds will be concentrated in an interface, and consequently Overton would be correct in postulating a lipid layer. On the other hand, proteins are likewise efficient depressors of interfacial tension and on theoretical grounds it is impossible to picture an interface between protoplasm and either water or a salt solution where an increased concentration of proteins, as well as fats and lipids, is absent. Accordingly Overton must be only partly correct in his lipid theory, and *the plasma membrane, from a purely mechanistic standpoint, must contain any and all of the constituents of protoplasm which effect a lowering of the surface energy at an interface.* Such a mechanistic picture of the plasma membrane would consist of a more or less completely denatured (surface-energy coagulated) protein gel, probably in the form of a fibrillar structure with fats, soaps, and lipids immeshed in the protein network. The transfer of lipid-soluble materials would be through the fat-soap-lipid portion of the structure, whereas the passage of water and such water-soluble materials as actually do pass in and out of the cells would be through the hydrated filaments of the protein network.

If a semi-permeable membrane has the structure noted above, the transference of water across the membrane, such as occurs in osmotic phenomena, can be considered to be due, at least in part, to an imbibition gradient across the membrane. Let us assume, for example, pure water on one side of such a membrane and a salt solution on the opposite side. Proteins have a lowered imbibition capacity when in contact with a salt solution. Accordingly we would expect the protein fibrils in contact with the salt solution to have a decreased water content, whereas those fibrils on the opposite side of the membrane in contact with pure water or with a solution of lower concentration would

be expected to have a higher water content. This would provide a hydration gradient across the fiber, and the normal process of diffusion in re-establishing an equilibrium within the protein fiber would cause a transfer of water from the point of lower salt concentration to the point of higher salt concentration. This suggestion was first made by Graham,[91] who wrote, "It now appears to me that the water movement in osmose is an affair of hydration and of dehydration in the substance of the membrane or other colloid septum. . . . The equilibrium of hydration is different on the two sides of the membrane of an osmometer. The outer surface of the membrane being in contact with pure water tends to hydrate itself in a higher degree than the inner surface does, the latter surface being supposed to be in contact with a saline solution. When the full hydration of the outer surface extends through the thickness of the membrane and reaches the inner surface, it there receives a check. The degree of hydration is lowered, and water must be given up by the inner layer of the membrane, and it forms the osmose."

Such a viewpoint attributes more than a passive role to the membrane, and though the ultimate equilibrium is determined by the relative concentrations on the two sides of the membrane, the membrane itself, according to the above hypothesis, plays a definite role in osmotic phenomena.

The transfer of fluids from the digestive tract to the body tissues and the transfer of liquid from the body tissues to the digestive tract involve both imbibition and syneresis. A normal individual secretes from 700 to 1000 cc. of saliva per day, from 600 to 900 cc. of bile, from 600 to 800 cc. of pancreatic juice, from 1000 to 2000 cc. of gastric juice, whereas the water intake is usually only from 1000 to 1500 cc. Accordingly there passes into the digestive tract from 3.9 to 6.2 liters per day or from 4 to 6 times the amount of liquid that is taken in the form of liquid food, indicating that the water which is absorbed in the digestive tract is used several times over in the form of liquids which are secreted into the digestive tract. The passage of this relatively enormous volume of fluids from the digestive tract into the blood stream cannot be adequately accounted for by pure osmosis, since in many instances the contents of the digestive tract have a higher osmotic pressure than the blood serum.

Imbibition, however, can account for this transfer of liquid. The proteins of the intestinal wall take up the water from the intestinal tract and transfer it to the proteins of the blood stream, which in turn carry it to other portions of the body where their imbibitional capacity is lowered and where they yield the water to other tissues or glands. The crystalloids which pass from the digestive tract to the blood stream

[91] Graham, Thomas, Liquid Diffusion Applied to Analysis, *Phil. Trans.* (1861), pp. 183–224.

and from the blood stream to the various cells and tissues of the body can be regarded as diffusing through a swollen gel rather than moving by osmotic processes across a membrane. Those chemicals which favor swelling of proteins favor absorption from the intestinal tract, and those substances which hinder the swelling of proteins hinder or prevent absorption from the intestinal tract. Magnesium sulfate more or less inhibits protein imbibition, and the effect of magnesium sulfate as a saline cathartic is to a large extent due to the prevention of the absorption of liquid from the intestinal tract, retaining the liquid within the tract. Similarly, agar, which furnishes bulk in cases of chronic constipation, is efficient because of its very pronounced hydrophilic nature, holding the water by imbibition forces against the pull of the intestinal wall and of the blood stream.

THE IMBIBITION OF LYOPHILIC COLLOIDS AS RELATED TO MEDICAL PROBLEMS.—It would be beyond the scope of this discussion to more than mention certain of the pathological problems in which lyophilic colloid-water relationships appear to be involved. The work of Martin Fischer on edema and nephritis has already been casually referred to. Fischer has ably defended his viewpoint that edema and nephritis are diseases characterized by abnormal imbibition of the body colloids. He notes that in the normal organism the degree of imbibition of the tissues is very finely regulated. For example, the brain may swell as much as 1000 per cent if removed from the body and placed in various solutions. If, however, the brain were to swell 3 per cent in the living organism, the volume of the brain would become greater than the volume of the skull cavity, and the pressure of the brain on the skull cavity would produce at first intense pain, followed by death.

The role of the salt content in the blood and tissues is at least in part the repression of the imbibition which would take place in the absence of such electrolytes. Bottazzi [92] suggests that the function of sodium chloride in the blood is to decrease the viscosity of the blood. If the inorganic salt content of the blood were decreased, as it sometimes is by excessive perspiration, the hydration of the blood proteins would be increased, and accordingly the viscosity of the blood likewise would be increased.

This problem of excessive perspiration with the loss of relatively large quantities of sodium chloride from the body may have a rather important bearing on industry. Moss [93] notes that, under muscular exertion and relatively high temperature, the sodium chloride content of perspiration ranges from 0.118 to 0.325 per cent, averaging 0.224,

[92] Bottazzi, F., Ricerche sopra soluzioni di colloidi organici, *Arch. fisiol.*, 7: 579–637 (1909).

[93] Moss, K. N., Some Effects of High Air Temperatures and Muscular Exertion upon Colliers, *Proc. Roy. Soc.* (*London*), 95B: 181–200 (1923).

and that under such conditions more sodium chloride may be lost through perspiration than in the urine. He suggests that workers in hot mines need more highly salted foods.

In certain of the very deep mines where the temperature regularly exceeds 100° F. a very considerable proportion of the miners developed intense headaches, necessitating hospital treatment at frequent intervals. In these mines the miners worked practically nude, drank large quantities of water, and owing to the high temperatures and humidities were continuously bathed in perspiration. It occurred to the physician in charge that possibly the headaches might be attributed to an excessive loss of sodium chloride through the perspiration, causing an excessive imbibition of the body tissues, including the nervous tissue. Accordingly the recommendation was made that, instead of the usual supply of water, the miners be furnished drinking water containing salts in approximately the proportion of a physiological salt solution. The effect of such substitution was striking in the extreme. The epidemic of headaches, which had persisted for years, disappeared as if by magic, indicating that the excessive loss of salts in the perspiration had been the determining factor.

Thomas and Andrews [94] point out that the serum proteins from edematous individuals have a greater affinity for water than those from normal individuals. Normal sera never swell more than 9 per cent, whereas sera from sufferers from uremia and edema swell enormously, often exceeding 50 per cent. They suggest that these observations be made a method of diagnosis.

A similar method of diagnosis is used in certain hospitals, the technic being to inject small amounts of water intradermally, noting the length of time required for the absorption of the water by the tissues. An extremely rapid absorption is characteristic of edematous individuals or of individuals who will shortly develop edemas, and such technic has permitted the detection of the onset of edema considerably in advance of the time when it could be observed by the usual clinical methods.[95, 96]

McQuarrie [97] made the important discovery that at least certain

[94] Thomas, W. A., and Andrews, E., A New Dialysis Test for Tissue Thirst, *Proc. Soc. Exptl. Biol. Med.*, 25: 773–774 (1928).

[95] McClure, W. B., and Aldrich, C. A., Time Required for Disappearance of Intradermally Injected Salt Solution, *J. Am. Med. Assoc.*, 81: 293–294 (1923).

[96] Aldrich, C. A., and McClure, W. B., The Intradermal Salt Solution Test. II. Its Prognostic Value in "Nephritis" with Generalized Edema, *J. Am. Med. Assoc.*, 82: 1425–1428 (1924). *Cf.* also *J. Am. Med. Assoc.*, 83: 1566–1567 (1924); 84: 1258–1259 (1925); *Arch. Internal Med.*, 37: 281–290 (1926); 41: 102–211 (1928); *Surg., Gynecol. Obstet.*, July, 1926, pp. 40–45.

[97] McQuarrie, I., The Significance of the Water Metabolism in Health and Disease (122 refs.), *J. Pediatrics*, 3: 539–567 (1933); Epilepsy in Children. The Relationship of Water Balance to the Occurrence of Seizures, *Am. J. Diseases Children*, 38: 451–467 (1929); (with D. B. Peeler), The Effects of Sustained Pituitary Anti-

types of epilepsy in children are either caused by, or are associated with, a disturbed water balance, and he has had remarkable success in the prevention of "grand mal" seizures by withholding water from the patient so that the patient becomes somewhat dehydrated. Certain patients who came to the hospital having forty or more grand mal seizures per day were freed of seizures after having been dehydrated to a point where they had lost 1–2 kilos of body water. If water was then returned to the diet, or if the antidiuretic hormone of the pituitary was given to such patients, they promptly went into a positive water balance with a recurrence of the seizures. However, if sufficient urea was dissolved in the ingested water, positive water balance would not take place, and seizures did not develop. With the dehydrated epileptic children typical grand mal seizures recurred when the antidiuretic hormone was given and water was taken at the rate of 2 to 5 cc. per kilo of body weight per hour. Under similar technic normal children did not develop epileptic seizures. McQuarrie does not believe that the epileptic seizures are the resultant of a brain edema but finds that the seizure is correlated with a marked negative potassium balance and a striking increase in the K: Na ratio in the urine, indicating that there is an apparent "leakage" of potassium from the cells of the nervous tissue. He suggests that perhaps the epileptic state is characterized by an inherent defect in the mechanism regulating the permeability of the brain cell membranes and that, at the same time when potassium leaks out, abnormal amounts of water and perhaps sodium ions pass into the cells of the nervous tissue. That there may be a shift in the water relationships of the brain is supported by the observations of Barbour[98] that during anesthesia the cerebrum loses water and the medulla gains water and that the shift appears to be directly associated with the phenomena of narcosis.

The problem of the nature of complement has interested immunologists for many years. Complement is some property or some component of normal serum which is destroyed by heating to 55° C. That this change may be in part colloid-chemical is suggested by the findings of du Noüy,[99] who studied the effect of heat on the viscosity of

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diuresis and Forced Water Drinking in Epileptic Children, A Diagnostic and Etiologic Study, *J. Clin. Investigation*, 10: 915–940 (1931); (with R. C. Manchester and C. Husted), Study of the Water and Mineral Balances in Epileptic Children. I. Effects of Diuresis, Catharsis, Phenobarbital Therapy and Water Storage, *Am. J. Diseases Children*, 43: 1519–1543 (1932); and Some Recent Observations Regarding the Nature of Epilepsy, *Ann. Internal Med.*, 6: 497–505 (1932).

[98] Barbour, H. G., Brain Water Movement during Anesthesia, *Science*, 73: 346–347 (1931).

[99] du Noüy, P. L., Études sur la viscosité du sérum sanguin en fonction de la température et sur l'hydratation de ses protéines, *Ann. Inst. Pasteur*, 42: 742–769 (1928).

normal rabbit serum. The viscosity fell during the temperature rise from 24.2° to 55° C. at the same rate as the fall in viscosity of water over a similar temperature range. The relative viscosity of the serum then began to increase sharply. When the hydration capacity of the serum proteins was calculated by equation (8) each gram of protein was found to occupy 2.09–2.19 cc. over the range of 24.2° to 55° C. At 57° the volume had increased to 2.58 cc., at 59° to 2.90 cc., and at 62° to 3.37. When held for 4 hours at 60°, the volume was 3.88 cc. or a hydration capacity 169 per cent of the normal. Du Noüy notes that this is an irreversible change, the protein micelles becoming hydrated at the expense of the water in the serum.

The field of pathology offers a great opportunity to the colloid chemist who is interested in the water relationships of the lyophilic colloids.

THE LIESEGANG PHENOMENA.—Liesegang[100] first described the peculiar reactions which take place when certain chemical processes are carried out in colloid gels. If potassium chromate is dissolved in a gelatin gel and a solution of silver nitrate is allowed to diffuse into the gel, the silver chromate which is formed is not dispersed uniformly throughout the gel but rather separates in a series of concentric rings, separated by more or less clear portions of the gel. Similarly, if potassium chromate is dissolved in sodium silicate and then the liquid acidified so as to form a silicic acid gel, when copper sulfate diffuses into such a gel, banded precipitates of copper chromate will be produced. Such banded precipitates are known as Liesegang rings.

In other instances, for example, in the reaction between potassium iodide and lead acetate, bands are not formed, but rather the lead iodide slowly separates in the form of very large crystals. Figure 109 illustrates the formation of copper chromate rings in a silicic acid gel, and the large crystals of lead iodide, likewise in silicic acid gel.

Much has been written in regard to this phenomenon, and numerous theories have been proposed. It is extremely difficult to evaluate the theories, and we still lack exact data by which one or the other of the theories can be proved.

It has been suggested that the silver ions, diffusing into a chromate-gelatin gel, form silver chromate which remains in a supersaturated state, owing to the protective action of gelatin. As the concentration of the silver increases, the silver chromate micelles are coagulated and crystallize out in the gel, forming a more or less impermeable layer. This silver chromate band would adsorb silver ions, retarding the diffusion of such ions through the gel, but would not adsorb nitrate ions which would pass through, possibly in the form of nitric acid, and such ions would advance ahead of the silver ions. As the silver

[100] Liesegang, R. E., Über einige Eigenschaften von Gallerten, *Naturw. Wochschr.*, 11: 353–362 (1896).

chromate gel ages, it would break down, become coarser in structure, allow the silver ions to pass through, and eventually a new point of supersaturation with subsequent precipitation would be reached, causing the formation of a new band.

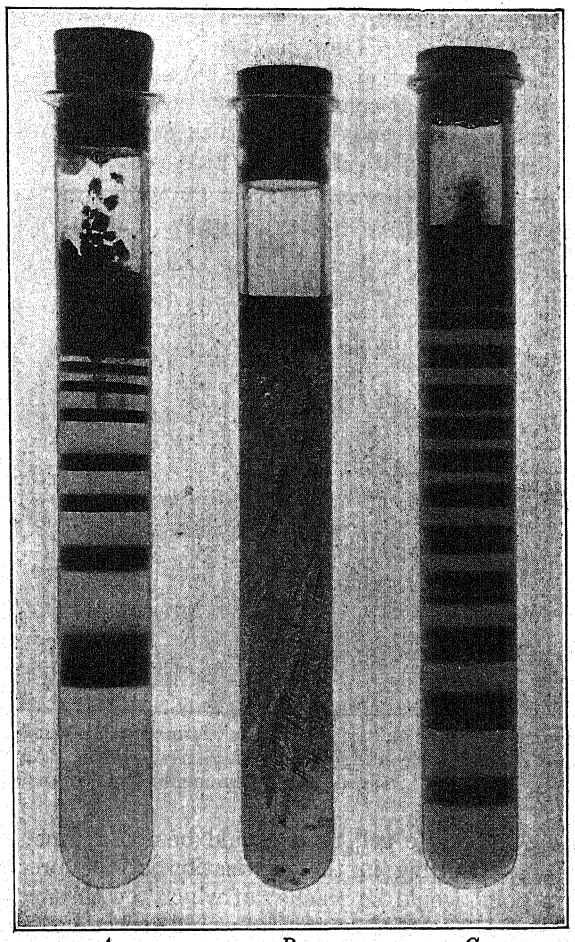


FIG. 109.—Liesegang phenomena. *A* and *C*, copper chromate in silica gel; *B*, lead iodide crystals in silica gel.

Such a theory, however, does not explain the specificity of the gel structure. Banded precipitates will be formed in a number of instances in silicic acid gels which are not formed in gelatin gels. Similarly, banded precipitates are formed in other instances in gelatin gels,

whereas no Liesegang rings are formed by the same reaction in silica gel. The nature of the gel in which the precipitation takes place plays an important role, and no theory as yet proposed accounts for the specificity of the gel.

It appears possible that the reaction may be due to differences in concentration of the reacting ions which bring about progressive peptization and coagulation. Thus, in Fig. 5 we have shown that silver bromide is held in colloidal dispersion by an excess of either silver or bromide ions, and that complete precipitation occurs only when the concentrations of silver and bromide ions are approximately equivalent. One could postulate, therefore, that in the diffusion of silver

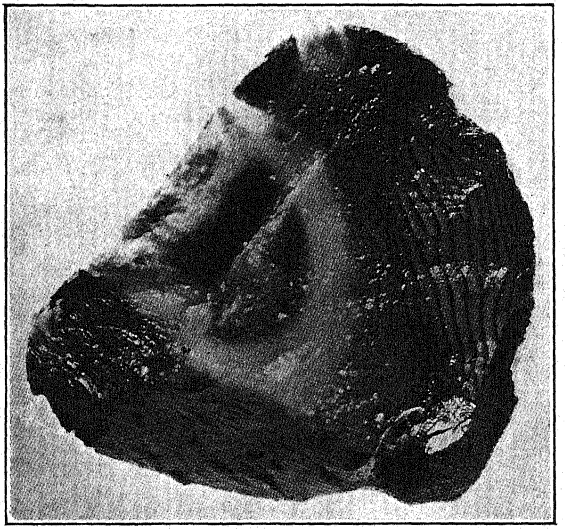


FIG. 110.—A photograph of an "artificial agate" formed by allowing copper sulfate to diffuse into a silica gel containing potassium ferrocyanide. An example of a Liesegang ring phenomenon.

nitrate into a chromate gel, there would be for a time an excess of chromate ions, resulting in the peptization of the precipitate until eventually a point would be reached at which the chromate and silver ions are equivalent. At this point one would expect a precipitate to be formed, making a band of silver chromate. A repetition of this process would account for succeeding bands. Here again, however, the specificity of the gel is not explained.

Liesegang has suggested that the bands characteristic of agates are due to the Liesegang phenomena. This appears to be so. Figure 110 is the photograph of an artificial agate produced under laboratory conditions by allowing copper sulfate to diffuse into a silicic acid gel containing potassium ferrocyanide. The silicic acid gel was allowed to set

in a collodion bag. The collodion bag was immersed in a dilute solution of copper sulfate and after several weeks was removed and the ball of gel cut in two. It will be noted that the laboratory product resembles the natural agate exactly. Bhatnagar and Mathur [101] have prepared such artificial agates and slowly dried them under pressure, in this way obtaining preparations which have a hardness of approximately 5.0 on the mineralogical scale and which can be cut and polished so as to make them practically indistinguishable from natural agates.

It frequently happens that precipitates which are not in exact rings form in gels. Under such conditions it almost invariably happens that the figures which do form are very symmetrical, *i.e.*, a line drawn down the center of the figure will yield two halves which are mirror images of each other. This problem of symmetry of structures formed in gels indicates relationships which may be of biological importance. However, here again no adequate explanation has been offered to account for the production of symmetrical figures.

Whether or not the Liesegang phenomena play a role in living processes is still an open question. Liesegang has suggested that the coloration patterns on butterfly wings, the stripes which occur on elytra of beetles, etc., may be due to these phenomena. Such explanations appear probable but are still unproved. Many interesting and beautiful museum specimens may be prepared by causing precipitates to form in the various gels. For those who are interested in following the literature in this field certain references are noted.[102-111]

[101] Bhatnagar, S. S., and Mathur, K. K., Studien über Bandstrukturen. Die Synthese gebänderter Steine, *Kolloid Z.*, 30: 368–371 (1922).

[102] Hatschek, E., The Liesegang Phenomenon, Second Report on Colloid Chemistry, pp. 21–26, Brit. Assoc. Advancement Sci., London (1918).

[103] Holmes, H. N., Experiments in Rhythmic Banding, *J. Am. Chem. Soc.*, 40: 1187–1195 (1918).

[104] Davies, E. C. H., Liesegang Rings. III. The Effect of Light and Hydrogen-Ion Concentration on the Formation of Colloidal Gold in Silicic Acid Gel. Rhythmic Bands of Purple of Cassius, *J. Am. Chem. Soc.*, 45: 2261–2268 (1923).

[105] Bradford, S. C., Adsorptive Stratification in Gels, *Biochem. J.*, 10: 169–175 (1916).

[106] Bradford, S. C., Adsorptive Stratification in Gels, II., *Biochem. J.*, 11: 14–20 (1917).

[107] Ganguly, P. B., Studies on the Formation of Liesegang Rings, *J. Phys. Chem.*, 31: 481–495 (1927).

[108] Schleussner, C. A., Diffusionvorgänge in Gelatine, II. Beiträge zur Kenntnis des Liesegang'schen Phänomens, *Kolloid Z.*, 34: 338–341 (1924).

[109] Handovsky, H., and du Bois-Reymond, E., Die Reaktion von Alkaloiden mit jodhaltiger Gelatine, *Kolloid Z.*, 33: 347–348 (1923).

[110] Bolam, T. R., and MacKenzie, M. R., The Influence of Lyophilic Colloids on the Precipitation of Insoluble Salts, Gelatin, and Silver Chromate, Pt. I and Pt. II, *Trans. Faraday Soc.*, 22: 151–177 (1926).

[111] Hedges, E. S., Liesegang Rings and Other Periodic Structures, 122 pp., Chapman and Hall, Ltd., London (1932).

So far as the author is aware only one study has dealt directly with an attempt to apply the Liesegang phenomenon to biochemical or medical problems. This is a study by Bucher [112] on the Liesegang phenomenon in blood plasma gels. Citrated blood plasma from a great variety of sources was mixed under rigidly controlled conditions with potassium bichromate solution and the mixture allowed to gellate in special test tubes. The diffusion of silver nitrate into such gels then resulted in the formation of Liesegang rings. The time required for each ring to form and the distance separating the rings was then recorded. A marked individuality was found for these data, blood serum from one individual being sharply differentiated from that of another individual. Characteristic species differences were also observed. Within a species there were apparently characteristic differences between young and old individuals and between normal and pathological individuals of the same age. Serums from individuals with carcinomas apparently gave different reactions from normal serums. Perhaps the most striking differences observed were those existing between normal cattle blood sera and the sera of cattle fetal blood. In the former typical sharply defined Liesegang rings formed, in the fetal blood gels, however, no rings formed but instead characteristic "flame-like" precipitations were produced. Repeated hemorrhages likewise produce characteristic changes in the pattern so that eventually no rings formed in the sera from the later bleedings. No explanation is available for the peculiar behavior which was observed, but unquestionably these observations will stimulate further investigation of the biochemical factors which are involved.

[112] Bucher, R., Die Diffusionsanalyse am Blutplasmagel, Benno Schwabe and Co., Basel (1937), 123 pp. 70 figs., many colored plates. (This paper appeared as Suppl. II to *Helv. Medica Acta,* 4, Heft 5 (1937).)

CHAPTER X

OSMOTIC PRESSURE AND ELECTRICAL CONDUCTIVITY

It is necessary to diverge again from a discussion of the phenomena which are characteristic of truly colloidal systems and to consider briefly two properties of true solutions, *i.e.*, osmotic pressure and electrical conductivity.

OSMOTIC PRESSURE

Diffusion and osmosis are of extreme importance in living processes. The passage of gases from the atmosphere to the plant or animal cells, or from one cell to another, the passage of ions and molecules from the soil solution into the root hairs of the plants and the transfer of these ions or molecules throughout the plant, or from animal cell to animal cell are all controlled by either diffusion or osmosis.[1] We have already seen that Thomas Graham throughout his period of active research studied practically only one natural phenomenon, the phenomenon of diffusion, and from those studies arose his concepts of the colloidal state of matter.

DIFFUSION IN GASES.—If we bring two different gases into a vessel, taking precautions not to mix them, we will find after a time that there is a perfectly uniform mixture throughout the vessel. If the two gases are separated by a membrane permeable to both, they will pass through the membrane in both directions, so that at equilibrium the mixture will have the same composition on both sides of the membrane. This process is called diffusion, regardless of whether or not a septum divides the two components.

We can differentiate between two kinds of diffusion: (1) diffusion where gases are separated by a membrane in which neither gas is soluble, and (2) diffusion through a moist membrane, the gases being more or less soluble in the liquid filling the pores of the membrane.

In the first case, a membrane might consist, for example, of a sheet of porous porcelain. *The undissolved gases diffuse at a rate which is inversely proportional to the square root of the density of the gas, pressure and temperature being held constant.* This is Graham's law.[2] If hydrogen, with a density of 1, is on one side of such a membrane,

[1] Lucké, B., and McCutcheon, M., The Living Cell as an Osmotic System and Its Permeability to Water, *Physiol. Rev.*, 12: 68–139 (1932) (131 refs.).

[2] Graham, T., On the Law of the Diffusion of Gases, *Phil. Mag.*, 2: 175–190, 269–276, 351–358 (1833).

and oxygen, with a density of 16, is on the other side of the membrane, the rates of diffusion will be proportional to $\sqrt{1}$ and $\sqrt{16}$, oxygen, therefore, having approximately one-fourth of the rate of diffusion of hydrogen. Since the rate of diffusion is unequal, it is possible to build up in such a system a pressure during the diffusion process. For example, if equal volumes of hydrogen and oxygen are placed on opposite sides of a porous membrane, as in *A*, after a definite period of time their distribution will be as at *B*. It will be noted that in *B* there are 2½ volumes of gas on one side of the membrane and 5½ volumes on the opposite side. Consequently, a pressure gradient has been set up. At *C*, however, representing equilibrium conditions, this pressure gradient has disappeared.

4 vols. H_2	4 vols. O_2 →	2 vols. H_2	2 vols. H_2	→ 2 vols. H_2	2 vols. H_2
		½ vol. O_2	3½ vols. O_2	2 vols. O_2	2 vols. O_2
A		*B*		*C*	
Initial State		Intermediate State		Equilibrium	

In the second case a moist membrane, in which the gases are soluble, separates the two gases, the densities of the gases play no part, and the rate of diffusion is directly proportional to the coefficient of solubility of the gases in the solvent. Carbon dioxide, which has a density of 44, will pass through a moist membrane much more rapidly than oxygen will since carbon dioxide is appreciably soluble in water, whereas the reverse conditions would hold for diffusion through a dry membrane.

The above relationships are of great importance in living processes. The passage of carbon dioxide from the atmosphere into the plant cells where photosynthesis takes place is controlled by both types of diffusion. Diffusion through the stomatal openings is largely the diffusion of undissolved gases and is inversely proportional to the density of the gas. As soon, however, as the gas passes the stomatal opening, it comes in contact with moist cell membranes, and its further passage into the cell and through the cells depends upon its solubility in the liquid phase. Gases, accordingly, move through cell walls and through protoplasm not in the gaseous state but in solution, and the greater the water content of the tissues or the membranes through which diffusion must take place, the more rapidly will diffusion occur.

Diffusion in Liquids.—The diffusion of molecularly dispersed material in liquid systems is approximately in accordance with the gas laws. Boyle's law states that *the volume of a fixed mass of a given gas at constant temperature is inversely proportional to the pressure.*

$$V = K\left(\frac{1}{P}\right) \tag{136}$$

Consequently,

$$V_1P_1 = V_2P_2 = K \tag{137}$$

which states that *the product of the simultaneous values of the pressure and the volume for a fixed mass of gas at constant temperature is constant.*

Avogadro's hypothesis states that *equal volumes of gases at the same temperature and pressure contain an equal number of molecules.*

Charles' law states that *the volume of a fixed mass of a gas at constant pressure is increased by 1/273 of its volume at 0° C. for each degree's rise in temperature.* Consequently we may write

$$PV = NRT \tag{138}$$

or,

$$P = \frac{NRT}{V} \tag{139}$$

an equation defining the pressure which will be exerted in a given system by a given mass of gas at a given temperature.

Dilute solutions obey the gas laws approximately, provided that (V) in equations (138) and (139) is evaluated as equal to the volume of the solvent and not the volume of the system. Consequently since a gram molecule of gas occupying a volume of 1 liter exerts a pressure of approximately 22.4 atmospheres, a gram molecule weight of solute in 1,000 cc. of solvent will exert an *osmotic pressure* of approximately 22.4 atmospheres. Diffusion in liquids is proportional to the difference in concentration, and at equilibrium there would be a uniform concentration of the solute existing throughout the system.

THE MEASUREMENT OF OSMOTIC PRESSURE.—In studies involving the osmotic pressure of aqueous solutions it is important to distinguish between *weight molar solutions* (1 gram molecule of solute in 1,000 grams of water) and *volume molar solutions* (1 gram molecule of solute in 1 liter of solution). In the former only will the correct values be approximated.

Various methods have been proposed for the measurement of osmotic pressure of aqueous solutions.

1. THE DIRECT METHOD.—The direct measurement was first used by Pfeffer [3] and was later modified by Morse and co-workers.[4, 5] In this method a semi-permeable membrane separates the solution from pure water. A semi-permeable membrane is permeable only to water and not to the ions or molecules contained in the solution under inves-

[3] Pfeffer, W., Osmotische Untersuchungen Studien zur Zellmechanik, Leipzig (1877).

[4] Morse, H. N., and Horn, D. W., The Preparation of Osmotic Membranes by Electrolysis, *Am. Chem. J.*, 26: 80–86 (1901).

[5] Morse, H. N., and Frazer, J. C. W., The Preparation of Cells for the Measurement of High Osmotic Pressures, *Am. Chem. J.*, 28: 1–23 (1902).

tigation. Morse and Frazer used a copper ferrocyanide gel precipitated in the pores of a porous porcelain cup. The gel which is used for a membrane must be solvated or wetted by the liquid of the solution. As already noted under gels, the transfer of water across such a membrane may be due in part to imbibitional forces. In Morse and Frazer's experiments the solution was placed inside the prepared porcelain cup, the entire apparatus was placed in contact with water, and the hydrostatic pressure which was reached by the diffusion of water through the membrane toward the more concentrated solution was measured on a mercury manometer.

The Earl of Berkeley [6] modified this technic by placing the solvent inside the cell and applying pressure to the external solution so as to keep the volume constant. This modification provides for a more rapid measurement and also yields more accurate values, inasmuch as the solution is not diluted by the passage of liquid through the semipermeable membrane.

2. INDIRECT METHODS.—(*a*) *The Plasmolytic Method.*—Nägeli, and in 1884 De Vries,[7, 8] studied the plasmolytic method as a means of measuring osmotic pressure in plant tissues. In this method the tissue is sectioned so as to be only a few cells in thickness, and these sections are then placed in a graded series of solutions of known concentration. Microscopic observations made at intervals show that, in certain of the solutions, liquid is withdrawn from the plant cell, the protoplasmic contents shrinking away from the cell wall. Such an appearance indicates that the solution in which the cells are immersed is of a higher osmotic pressure than the solution within the plant cell. Such solutions are called *hypertonic* solutions. In other solutions it will be noted that the plant cell absorbs water, becomes larger, and may eventually burst. This indicates that water is being absorbed by the plant cell from a solution which has a lower osmotic pressure than the cell contents. Such solutions are *hypotonic.* Between these two extremes will be found some concentration of the external solution which will neither withdraw water from the plant cell nor yield water to the plant cell. These solutions are *isotonic* or *isosmotic* with the contents of the cell.

Figure 111 shows the appearance of filaments of *Spirogyra* undergoing plasmolysis in a 0.35 *M* cane sugar solution. At *A* is seen a cell in which the protoplasmic contents are still approximately normal. In cell *B* the protoplasmic contents have begun to draw away from the cellulose wall, owing to the transfer of liquid from the cell toward a

[6] Berkeley, Earl of, and Hartley, E. G. J., A Method of Measuring Directly High Osmotic Pressures, *Proc. Roy. Soc.* (*London*), 73: 436–443 (1904).

[7] De Vries, Hugo, Eine Methode zur Analyse der Turgorkräfte, *Jahrb. wiss. Botan.*, 14: 427–601 (1884).

[8] De Vries, Hugo, Zur plasmolytischen Methodik, *Botan. Z.*, 42: 289–298 (1884).

hypertonic sugar solution. At cell *C* the protoplasmic mass has been so highly dehydrated as to contract into a rather small clot. Reference may be made to the paper of Miss Delf [9] for a consideration of the technic which must be employed in plasmolytic studies.

There are several objections to the use of the plasmolytic method. In the first place, the cell or cells which are observed may be abnormal and not representative of the plant as a whole, or they may have been injured in the sectioning operation. Another objection is that the solution in which the cells are placed may have a toxic action on the cell or may have a coagulating or peptizing action, thus changing the permeability of the cell membrane. Certain solutions exert a toxic action in the cell or the cell membrane, so that the plasmolytic results

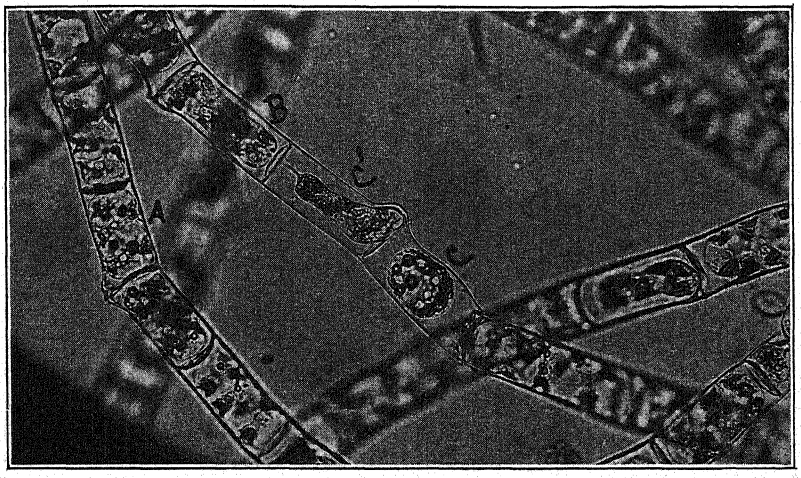


FIG. 111.—Plasmolysis of cells of Spirogyra in 0.35 *M* sucrose solution. Cell A is essentially normal; the plasmolysis increases progressively from B → C → C′ (Photograph, courtesy of Dr. Lloyd.)

which are obtained may not yield true osmotic pressure values. Solutions of potassium nitrate and solutions of cane sugar have been very generally employed in plasmolytic studies. Beck [10] draws the conclusion that cane sugar gives correct results for osmotic pressure but that potassium nitrate solutions do not.

Osterhout distinguishes between true and false plasmolysis and notes that dilute solutions or even water may cause plasmolysis, such

[9] Delf, E. M., Studies of Protoplasmic Permeability by Measurement of Rate of Shrinkage of Turgid Tissues. I. The Influence of Temperature on the Permeability of Protoplasm to Water, *Ann. Botany*, 30: 283–310 (1916).

[10] Beck, W. A., Cane Sugar and Potassium Nitrate as Plasmolysing Agents, *Protoplasma*, 1: 15–72 (1926).

as would normally be brought about by hypertonic solutions. He attributes this false plasmolysis to the coagulation of protoplasm, due to the entrance of water, and states that it is especially noticeable in studies dealing with marine plant cells.

Maximow [11] has suggested a macro method for studying the osmotic pressure of plant roots and root hairs. Figure 112 illustrates his experiment. The plant is grown in soil, and when it has reached the proper stage of growth, the soil is carefully washed away from the roots, care being taken not to mechanically injure the root structures. The entire plant is then immersed under water and the top cut off with a sharp knife. Connection is made between the cut portion of the stem and a bent glass tube by means of rubber tubing. The plant is then removed, and the roots placed in solutions of sucrose of varying concentration. When the roots are placed in hypotonic solutions, water is drawn through the plant cell walls by osmosis and liquid flows from the end of the glass tubing, as shown in Fig. 112. If, now, the plant is removed to a hypertonic solution, liquid is withdrawn from the root cells, and liquid not only ceases to flow from the tip of the glass tube, but actually recedes in the glass capillary. When the plant is placed in isotonic solutions, the liquid level remains stationary in the glass tube.

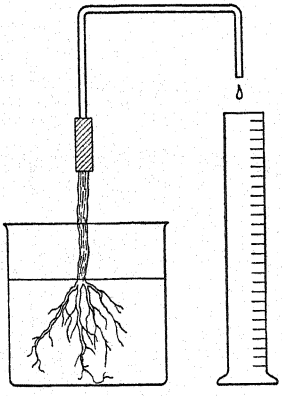


FIG. 112.—A diagrammatic representation of Maximow's method for determining the osmotic pressure of plant root cells. (In this instance, since there is an upward flow of liquid, the roots are in a hypotonic solution.)

Such a technic appears to afford a more accurate index of the osmotic pressure of roots than would the usual plasmolytic technic. There is little danger of mechanically injuring the roots, and the osmotic pressure obtained by the Maximow technic is the average osmotic pressure of the cell contents of the entire mass of roots, as determined under conditions very similar to the normal relations existing between a plant and the soil solution.

(*b*) *Vapor-pressure Methods.*—The vapor-pressure methods of studying osmotic pressure depend upon the phenomenon of isothermal distillation of solutions of lower concentrations to those of higher concentrations. In Fig. 113 is shown a closed system with water in the lower portion of the container and a tube of sugar solution suspended in the system, the top of the tube being open, the bottom being closed with a semi-permeable membrane. Water rises in the tube con-

[11] Maximow, N. A., Lecture, University of Minnesota, Oct. 26, 1926.

taining the sugar to the equilibrium point s' as the result of osmotic pressure. At this point the hydrostatic pressure of the solution in the tube which tends to drive the liquid downward through the semi-permeable membrane just balances the osmotic pressure of the sugar

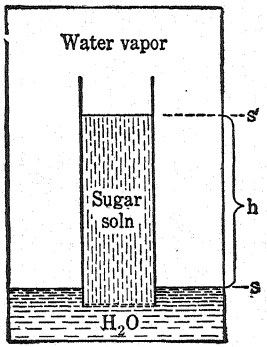


FIG. 113.

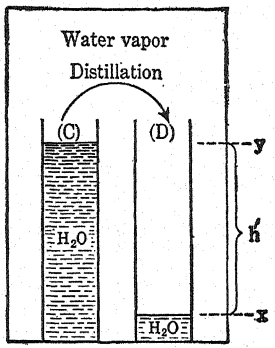


FIG. 114.

FIG. 113.—A diagrammatic representation of the relationship between vapor pressure and osmotic pressure.

FIG. 114.—A diagrammatic representation of isothermal distillation from a water surface at a higher level to one at a lower level.

solution, *i.e.*, the force which tends to draw water up through the membrane. The vapor pressure of the pure solvent must accordingly be greater than that of the sugar solution by an amount equal to the pressure of a column of water vapor of height h which is the difference in level between the two surfaces s and s'. Therefore,

$$\text{Vapor pressure at } s = (\text{vapor pressure at } s') + h \qquad (140)$$

Only under such conditions will an equilibrium be reached and no distillation of water vapor take place.

If we were to construct a closed system containing two vessels, each containing water the surface of which was at the different levels, x and y, as shown in Fig. 114, at *equilibrium* both vessels would contain water at the same level, *i.e.*, there would be isothermal distillation from the water in C to the water in D because the vapor pressure of the water in C at the surface of the water is greater than that in the vapor phase above D at a corresponding level in the closed system by an amount which is proportional to the weight of a column of water vapor of height h'. Of course, the *actual rate* of distillation would be very slow and might not be realizable experimentally within the usual time limits. The crucial point is that the system, shown in Fig. 114, is not in equilibrium.

In Fig. 115 we again have a closed system, inclosing two vessels, one containing pure water, the other a sugar solution. Here also distillation will be from the water to the sugar solution, and *at equilibrium* the beaker containing the water will be empty, all the water having been transferred to the sugar solution. The water vapor phase here acts as a semi-permeable membrane, permeable in one direction to water vapor but not in the other direction to the sugar molecules. Hence, there is a distillation from the point of high vapor pressure to that of lower vapor pressure.

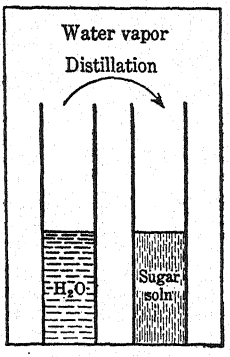


FIG. 115. — A diagrammatic representation of isothermal distillation from a solution of low osmotic pressure to one having a higher osmotic pressure.

Barger [12] used this method to determine the molecular weights of small quantities of organic compounds and Miss Halket [13] used it to determine the osmotic pressure of plant saps. In her experiments only small quantities of plant saps were available. She prepared capillary glass tubes of very fine bore and cleaned them so scrupulously that the sap or liquids would not adhere to the glass wall. She then drew a small quantity of the plant sap into the capillary tube, followed by a tiny bubble of air, then by a drop of sugar solution of known concentration; this in turn was followed by another bubble of air, a drop of sap, a bubble of air, a drop of stronger sugar solution, etc. This arrangement placed the drops of plant sap between drops of sugar solutions of progressively increasing concentration. *A sugar solution with an osmotic pressure greater than that of the sap caused distillation from the sap to the sugar solution, so that the volume of the plant sap droplet decreased. A sugar solution with an osmotic pressure lower than that of the sap caused distillation from the sugar solution to the plant sap, and the volume of the sap droplet increased. That droplet that was so situated as to remain practically constant in size was judged to be placed between two sugar solutions, one of which had a slightly greater osmotic pressure and one a slightly lower osmotic pressure than the plant sap.* In this way the osmotic pressure of very small quantities of solutions can be approximately determined. The increase or decrease in the volume of the droplets was measured by means of a micrometer eyepiece in a microscope.

Townend [14] devised an apparatus for making very exact osmotic

[12] Barger, G., A Microscopical Method of Determining Molecular Weights, *J. Chem. Soc.*, 85: 286–324 (1904).

[13] Halket, A. C., On Various Methods for Determining Osmotic Pressures, *New Phytologist*, 12: 164–176 (1913).

[14] Townend, R. V., A New Method for Measuring Osmotic Pressure, *J. Am. Chem. Soc.*, 50: 2958–2966 (1928).

pressure measurements by the vapor-pressure method, and as already discussed, Hill (*loc. cit.*) used vapor pressure in studying water relationships in blood and muscle tissues.

(*c*) *Elevation of the Boiling Point.*—If the vapor pressure is lowered with increasing concentration of solute, it must be self-evident that the boiling point must be raised. Accordingly, osmotic pressure may be calculated by the increase in the boiling point. The technic for this determination can be found in any good textbook on physical chemistry. It will not be discussed in detail here, since biological systems are so readily altered at high temperatures as to render this method of little use in dealing with biological fluids.

(*d*) *Depression of the Freezing Point.*—If there is an elevation of the boiling point and a decrease in vapor pressure due to an increased concentration of solute, solutions must freeze at a lower temperature than the pure solvent. Any adequate textbook on physical chemistry will afford directions for carrying out freezing-point determinations.

The depression of the freezing point is one of the most generally used methods available to the biologist for the study of osmotic pressures of biological fluids. In dealing with plant saps, Dixon and Atkins [15] pointed out in 1913 that representative samples of plant saps could not be obtained by simply pressing the fresh tissues to extract the sap, inasmuch as the cell walls acted as ultrafilters, retaining a greater or less proportion of the solutes in the sap.

These observations have been confirmed,[16] and it was found that, when plant leaves are pressed without a preliminary freezing, saps may in some instances be obtained which are more dilute than the saps within the cells, or in other instances more concentrated than such saps. Dixon and Atkins suggest freezing the tissues in liquid air and then thawing them, thus making the cell membranes permeable so that uniform and representative samples of cell sap can be obtained. Gortner and Harris [17] found that a preliminary freezing in an ice and salt mixture answered the same purpose. In this paper the details of the method for the determination of the osmotic pressure of plant saps are given. Later [18] the values for the depression of the freezing point

[15] Dixon, H. H., and Atkins, W. R. G., Osmotic Pressures in Plants. I. Methods of Extracting Sap from Plant Organs, *Sci. Proc. Roy. Dublin Soc.* (n.s.), 13: 422–433 (1913). Also, *Notes from Botan. Sch. Trinity Coll. Dublin*, 2:154–172 (1913).

[16] Gortner, R. A., Lawrence, J. V., and Harris, J. A., The Extraction of Sap from Plant Tissues by Pressure, *Biochem. Bull.*, 5: 139–142 (1916).

[17] Gortner, R. A., and Harris, J. A., Notes on the Technique of the Determination of the Depression of the Freezing Point of Vegetable Saps, *Plant World*, 17: 49–53 (1914).

[18] Harris, J. A., and Gortner, R. A., Notes on the Calculation of the Osmotic Pressure of Expressed Vegetable Saps from the Depression of the Freezing Point, with a Table for the Values of P for $\Delta = 0.001°$ to $\Delta = 2.999°$, *Am. J. Botany*, 1: 75–78 (1914).

were tabulated in terms of osmotic pressure, using the equation

$$P = 12.06\Delta - 0.021\Delta^2 \tag{141}$$

where P = osmotic pressure;
Δ = *corrected* depression of the freezing point.

It is necessary to correct the observed depression of the freezing point in order to obtain the true depression. This is due to the fact that on freezing a certain amount of the water separates as ice. Accordingly, the system is then that of a more saturated solution than the original sap in equilibrium with ice crystals, and the observed depression of the freezing point is the freezing point of this more concentrated solution. If a liter of pure water is under-cooled to $-1°$ C. before ice crystallization begins, 1/80 of the water will separate in the form of ice, and when 1/80 has thus separated, the temperature of the liter of water will have risen to 0° C. This is due to the fact that the latent heat of fusion of ice is 80 calories. Consequently the crystallization of 1 gram of ice liberates 80 calories of heat. Using this value, one can easily correct the observed depression of the freezing point by means of the equation

$$\Delta = \frac{\Delta'\left(V - \frac{uV}{80}\right)}{V} \tag{142}$$

or

$$\Delta = \Delta' - 0.0125\, u\Delta' \tag{143}$$

where Δ = the corrected depression of the freezing point;
Δ' = the observed depression of the freezing point;
u = degrees of under-cooling before ice separation begins.

Either a Haidenhain thermometer, fixed to read from $+1°$ to $-5°$ C. in 1/100° subdivisions, or a sensitive thermocouple [19] may be used to advantage in measuring the freezing point of aqueous solutions, plant saps, or biological fluids in general.

Harris and his co-workers [20] made extensive studies of the osmotic pressure and other physicochemical properties of plant saps as related to plant distribution as determined by environmental conditions, and have shown that valuable ecological data may be secured from such studies. In 1920, they found in a Utah alkali spot a plant, *Atriplex nuttallii*, the sap of which had a depression of the freezing point of

[19] Wright, R. C., and Harvey, R. B., The Freezing Point of Potatoes as Determined by the Thermoelectric Method, U. S. Dept. Agr. Bull. 895 (1921).

[20] Harris, J. Arthur, The Physico-Chemical Properties of Plant Saps in Relation to Phytogeography, 339 pp., University of Minnesota Press (1934); "J. Arthur Harris, Botanist and Biometrician," edited by C. O. Rosendahl, R. A. Gortner, and G. O. Burr (209 pp.), University of Minnesota Press (1936).

14.4° C., corresponding to an osmotic pressure of approximately 173 atmospheres. Later Harris observed a similar depression in the sap of *Atriplex confertifolia*. As already noted in Chapter IX, the depression of the freezing point has been utilized by Newton and others in a technic for the determination of bound water in the presence of hydrophilic colloids.

ELECTRICAL CONDUCTIVITY

The determination of the osmotic pressure of solutions or of biological fluids yields information which can be correlated with the actual concentration of molecularly dispersed substances present in such solutions, but gives no information as to the relative proportion of electrolytes and non-electrolytes.

According to the theory of Arrhenius, electrolytes in aqueous solutions are more or less completely dissociated into ions, and the conductance of an electric current through a solution of an electrolyte is due to the presence of these ions.

By osmotic-pressure technic, each ion is found to contribute independently to the osmotic pressure of the system. Thus, assuming complete dissociation of sodium chloride, one gram molecule of sodium chloride would produce an osmotic pressure twice as great as one gram molecule of sucrose. The degree of ionization (or the "activity" of the ions) of electrolytes can be determined by making use of osmotic-pressure technic.

In dealing with biological fluids and tissues it is often desirable to differentiate between the total solutes in a system and the relative concentration of electrolytes and non-electrolytes. Such a differentiation can be secured, at least in part, by studying the electrical conductivity of such fluids or tissues, and such studies have been used extensively in biological investigations, particularly as a means of detecting changes in permeability of protoplasm or of the plasma membrane.

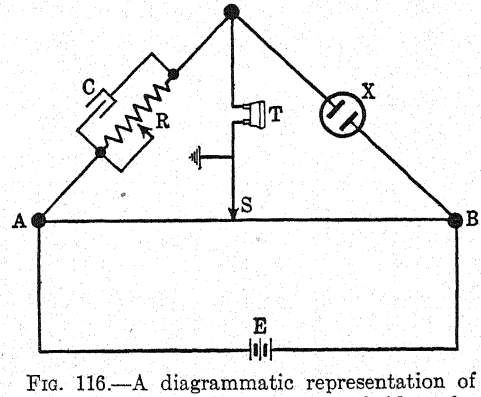


FIG. 116.—A diagrammatic representation of the conventional Wheatstone bridge for measuring electrical conductivity.

Figure 116 represents diagrammatically the apparatus necessary for the measurement of electrical conductivity. We have at R a variable resistance across which is shunted a variable air condenser C, in order to balance the capacity of the system. AB is the slide wire of the Wheatstone bridge, having

uniform diameter and electrical resistance. X is the electrolytic cell, containing platinized electrodes between which is inserted the tissue or fluid under investigation. A source of electric current E is connected to the two ends of the bridge, and an electric current detecting apparatus, such as a telephone or a galvanometer, is connected, as shown at T, to a sliding contact S attached to the resistance wire of the Wheatstone bridge. In such a set-up, when an electric current is impressed upon the system, the sliding contact is adjusted so that the current passing through the cell is exactly balanced by the variable resistance. The resistance of the material in the cell can then be calculated from the known value used at R and the ratio of the two arms of the Wheatstone bridge.

For biological tissues and solutions it is desirable to use an alternating current of rather high frequency and purity. Currents of 1000 cycles are generally employed, although recently currents of radio frequency up to 5,000,000 cycles per second are coming more and more into use. When such currents are employed, it is, of course, necessary to replace the current detector by radio apparatus.

Measurements of electrolytic conductivity can be made rapidly and relatively accurately. In general, the values are expressed in terms of specific electrical conductivity, conductivity being the reciprocal of resistance. Resistance is measured in ohms. Conductivity is expressed in mhos.

$$\frac{1}{\text{ohms resistance}} = \text{mhos conductivity} \qquad (144)$$

Anything which impedes an ion in its passage through a solution or tissue increases the resistance of that system, and accordingly decreases the conductivity.

The specific conductivity is the conductivity of a cube of solution, 1 cm. on an edge, between electrodes suspended exactly 1 cm. apart, each electrode having 1 sq. cm. of area. It is obvious that the construction of a conductivity cell having exactly these specifications would be extremely difficult. As a matter of fact, such construction is rarely attempted. Instead the "*cell constant*" of the cell is determined by using in the cell a solution, the specific electrical conductivity of which has already been determined, and from such data the cell constant is calculated from the equation

$$\frac{B}{A} R\kappa = \text{cell constant} \qquad (145)$$

where B = the length of the arm of the bridge between B and S in Fig. 116;
A = the length of the arm of the bridge between A and S in Fig. 116;
R = the resistance in ohms, as measured on the resistance box;
κ = the specific electrical conductivity of the known solution.

Generally, solutions of potassium chloride are used as standard solutions. Table L lists the specific electrical conductivity of various solutions of potassium chloride at various temperatures.

TABLE L

SPECIFIC CONDUCTIVITY OF STANDARD KCl SOLUTIONS FOR DETERMINING THE RESISTANCE-CAPACITY OF CONDUCTIVITY VESSELS

Temperature	Concentration of KCl Solution			
	$\frac{N}{1}$	$\frac{N}{10}$	$\frac{N}{50}$	$\frac{N}{100}$
° C.	κ	κ	κ	κ
0	0.06541	0.00715	0.001521	0.000776
1	0.06713	0.00736	0.001566	0.000800
2	0.06886	0.00757	0.001612	0.000824
3	0.07061	0.00779	0.001659	0.000848
4	0.07237	0.00800	0.001705	0.000872
5	0.07414	0.00822	0.001752	0.000896
6	0.07593	0.00844	0.001800	0.000921
7	0.07773	0.00866	0.001848	0.000945
8	0.07954	0.00888	0.001896	0.000970
9	0.08136	0.00911	0.001945	0.000995
10	0.08319	0.00933	0.001994	0.001020
11	0.08504	0.00956	0.002043	0.001045
12	0.08689	0.00979	0.002093	0.001070
13	0.08876	0.01002	0.002142	0.001095
14	0.09063	0.01025	0.002193	0.001121
15	0.09252	0.01048	0.002243	0.001147
16	0.09441	0.01072	0.002294	0.001173
17	0.09631	0.01095	0.002345	0.001199
18	0.09822	0.01119	0.002397	0.001225
19	0.10014	0.01143	0.002449	0.001251
20	0.10207	0.01167	0.002501	0.001278
21	0.10400	0.01191	0.002553	0.001305
22	0.10594	0.01215	0.002606	0.001332
23	0.10789	0.01239	0.002659	0.001359
24	0.10984	0.01264	0.002712	0.001386
25	0.11180	0.01288	0.002765	0.001413
26	0.11377	0.01313	0.002819	0.001441
27	0.11574	0.01337	0.002873	0.001468
28	.....	0.01362	0.002927	0.001496
29	.....	0.01387	0.002981	0.001524
30	.....	0.01412	0.003036	0.001552

In dealing with biological fluids or tissues, or even mixtures of electrolytes, it is impossible to translate specific electrical conductivity into concentration values. Accordingly, one cannot calculate from

electrical conductivity determinations the concentration of non-electrolytes which are present in a given biological system. As a rule, osmotic-pressure determinations are a more accurate index of the actual concentration of electrolytes and non-electrolytes than electrical conductivity is of the electrolyte content.

The use of the ratio κ/Δ has been suggested [21] as of value in indicating changes in the ratio of electrolytes to non-electrolytes in plant saps, and this ratio was used rather generally in Harris' studies of plant saps as related to geographical environment. It is not unusual to find plant saps, at least in certain halophytes, which contain inorganic chlorides exceeding the concentration of 100 grams of sodium chloride per liter (calculated as sodium chloride from the chloride content of the plant sap). Similarly, biological reactions always occur in solutions of mixed electrolytes. The elucidation of the physicochemical properties of such systems accordingly becomes of paramount interest to the biologist and to the biological chemist.

The presence of organic acids in biological fluids does not appreciably affect the electrical conductivity of those fluids. The explanation probably lies in the fact that organic acids are relatively slightly ionized. The electrical conductivity of the sap of the leaves of the gooseberry is very much greater than that of the fluids which can be expressed from the green berries. In general, leaf tissue fluids have a much higher conductivity than fluids which can be expressed from either green or ripe fruits.

In a study [22] of the relation between the osmotic concentration of the leaf sap and the height of leaf insertion in trees, it was noted that the osmotic pressure, as measured by depression of the freezing point, increased with the tree height at which the leaves were collected, whereas the specific electrical conductivity decreased from the lower to the higher levels. A part of the data is shown in Table LI. Evidently there is an increased photosynthesis in those leaves in the upper portion of a tree, as indicated by an increased osmotic pressure, and a decreased ratio of κ/Δ. Whether or not this increase is due to more favorable light conditions, or whether it is due to a more favorable ratio of the electrolytes present in the cell sap can be determined only by more extensive studies. We know that potassium in some way favors carbohydrate formation, and it may well be that ions retarding photosynthesis may be screened out in their passage through the conducting system, so that a more optimal mixture of inorganic ions is present at the higher levels. This suggestion is borne out by the ob-

[21] Harris, J. A., Gortner, R. A., and Lawrence, J. V., Studies on the Physicochemical Properties of Vegetable Saps, III. *Biochem. Bull.*, 4: 52–79 (1915).

[22] Harris, J. A., Gortner, R. A., and Lawrence, J. V., The Relationship between the Osmotic Concentration of Leaf Sap and Height of Leaf Insertion in Trees, *Bull. Torrey Botan. Club*, 44: 267–286 (1917).

TABLE LI

THE DEPRESSION OF THE FREEZING POINT (Δ), THE OSMOTIC PRESSURE IN ATMOSPHERES (P), THE SPECIFIC ELECTRICAL CONDUCTIVITY (κ), AND THE RATIO OF SPECIFIC CONDUCTIVITY TO DEPRESSION OF THE FREEZING POINT (κ/Δ), FOR THE TISSUE FLUIDS OF LEAVES AT DIFFERENT HEIGHTS OF LEAF INSERTION IN TREES

Tree	Height, ft.	Δ, °C.	P, atm.	$\kappa \times 10^5$, mhos	$\frac{\kappa}{\Delta} \times 10^5$
Acer rubrum.....	12	1.334	16.05	938	703
	27	1.359	16.35	911	671
	47	1.385	16.66	863	623
Juglans cinera.....	8	1.398	16.81	1332	954
	21	1.484	17.85	1197	807
	32	1.513	18.19	1174	777
	38	1.429	17.18	1218	852
	44	1.525	18.33	1114	731
	52	1.522	18.31	1046	687
Quercus palustris.....	9	1.681	20.21	1057	629
	23	1.728	20.77	1002	580
	33	1.932	23.22	903	467
Robinia pseudacacia....	24	0.915	11.01	1362	1488
	36	0.932	11.22	1267	1360
	45	0.969	11.66	1232	1272
Betula lenta.....	12	1.411	16.97	1160	822
	29	1.518	18.25	1056	696
Betula lutea.....	11	1.050	12.63	990	942
	25	1.173	14.11	1083	923
	39	1.257	15.12	1110	883
	52	1.331	16.01	1027	772
	66	1.239	15.55	1160	897

servation of Ayres [23] that the concentrations of N, P_2O_5, K_2O, CaO, and MgO are greater in the region of the growing point than in any other part of the plant, whereas the total ash content decreases progressively from the base to the growing point.

Electrical conductivity has been used very extensively in measuring changes in permeability. Osterhout in particular has extensively developed this field, and his [24] general summary may be consulted for a bibliography of the earlier literature.

[23] Ayres, A., Variation of Mineral Content of Sugar Cane with Age and Season, *Hawaiian Planters' Record*, 37: 197–206 (1933).

[24] Osterhout, W. J. V., Injury, Recovery, and Death, in Relation to Conductivity and Permeability, J. B. Lippincott Company, Philadelphia (1922).

Figure 117 shows the curves for electrical resistance of the sea alga, *Laminaria agardhii*, in electrolyte solutions having the same conductivity as sea water. It will be noted that the electrolytic resistance falls sharply in pure solutions of sodium chloride or magnesium chloride, whereas it rises sharply in solutions of calcium chloride, manganous chloride, and lanthanum nitrate. In the last three solutions the initial rise is followed by a subsequent fall in resistance.

Osterhout notes that all these curves are reversible in the initial part of the curve. Thus, the resistance may be appreciably decreased by immersing the cells in sodium chloride solution, and recovery will take place if the cells are transferred to sea water. If, however, the decrease of conductivity is too great, recovery is no longer possible. Similarly, the resistance may be allowed to increase in calcium chloride solutions, and recovery to normal will take place. There is, however, a critical point past which recovery is no longer possible. Osterhout regards the curves in either direction as indicative of a death process and believes that death changes may be studied quantitatively by changes in electrical conductivity. It is thus possible to injure an organism so that it behaves as though it were 5 per cent dead, 10 per cent dead, 20 per cent dead, etc., and its biochemical reactions under such conditions can be studied. Similar studies have been made with animal tissues, for example, frog skin, and similar effects have been observed.

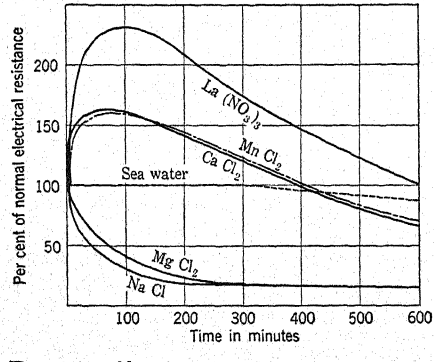


FIG. 117.—Showing the change in relative electrical resistance of *Laminaria agardhii* with time, in solutions having the same electrical conductivity as sea water. (Data of Osterhout.)

Clowes [25] interprets these results as changes in protoplasmic emulsions, due to the effect of the specific ions, regarding the plasma membrane as an emulsion fairly closely balanced between oil-in-water and water-in-oil types, the sodium chloride causing a more complete peptization of the cell membrane and forming a more perfect oil-in-water type, the calcium chloride causing inversion to a water-in-oil type. If water is the continuous phase, a low electrical resistance should result. If oil is the continuous phase, there would be a high electrical resistance. Eventually either calcium chloride or sodium chloride breaks

[25] Clowes, G. H. A., Protoplasmic Equilibrium. I. Action of Antagonistic Electrolytes on Emulsions and Living Cells, *J. Phys. Chem.*, 20: 407–451 (1916).

the emulsion, causing a separation of the two phases with the ensuing death of the organism. This theory appears to have experimental backing, inasmuch as emulsions soaked up in filter paper show, to a considerable degree, conductivity phenomena similar to those shown in Fig. 117. However, the form of the curves likewise suggests electrokinetic changes, and it may well be that the proteins of the cell wall and protoplasm are involved, as well as the fats and lipids.

More recent studies of the effect of electric currents of varying frequency on electrical conductivity of biological tissues have thrown some doubt on certain of the earlier studies of electrical conductivity in biological systems. McClendon [26-28] points out that very different values for electrical conductivity may be obtained when the frequency of the electric current is varied. However, Blinks,[29] studying *Laminaria* and using Osterhout's apparatus, concludes, "We may therefore hold to the interpretation advanced by Osterhout that the observed resistance change is really a change in the permeability of protoplasm to ions. Since the resistance may rise 60 per cent or more above the normal value in sea-water, it is evident that there must be considerable ionic exchange in the normal state."

Crile and his co-workers [30-33] used electrical conductivity to measure changes taking place in animal tissues, and from the experiments Crile developed an electrochemical theory of living processes.

It would take us too far afield to discuss adequately the data and theories which he presents. In many respects the experimental data

[26] McClendon, J. F., Colloidal Properties of the Surface of the Living Cell, Colloid Symposium Monograph, Vol. IV, pp. 224–233, Chemical Catalog Company, New York (1926).

[27] McClendon, J. F., Colloidal Properties of the Surface of the Living Cell, I. Conductivity of Blood to Direct Electric Currents, *J. Biol. Chem.*, 68: 653–663 (1926).

[28] McClendon, J. F., Colloidal Properties of the Surface of the Living Cell, II. Electric Conductivity and Capacity of Blood to Alternating Currents of Long Duration and Varying in Frequency from 260 to 2,000,000 Cycles per Second, *J. Biol. Chem.*, 69: 733–754 (1926). Cf. Remington, R. E., The High Frequency Wheatstone Bridge as a Tool in Cytological Studies; with Some Observations on the Resistance and Capacity of the Cells of the Beet Root. *Protoplasma*, 5: 338–399 (1928).

[29] Blinks, L. R., High and Low Frequency Measurements with *Laminaria*, *Science*, 68: 235 (1928).

[30] Crile, G. W., A Bipolar Theory of Living Processes, The Macmillan Company, New York (1926).

[31] Crile, G. W., An Electro-chemical Theory of Normal and Certain Pathological Processes, *Proc. Am. Phil. Soc.*, 60: 546–552 (1921).

[32] Crile, G. W., Hosmer, H. R., and Rowland, A. F., The Electrical Conductivity of Animal Tissues under Normal and Pathological Conditions, *Am. J. Physiol.*, 60: 59–106 (1922).

[33] Crile, G. W., The Phenomena of Life; A Radio-Electric Interpretation, 379 pp., W. W. Norton and Company, New York (1936).

with animal tissues are more or less analogous to the findings of Osterhout and others, using plant tissues. Crile finds that many substances alter the electrical conductivity of animal tissues and that, in general, exhaustion from any cause, such as surgical shock, insomnia, emotion (fright), infection, etc., is characterized by a diminished conductivity of the brain and nervous tissue, activation, on the other hand, being characterized by an increased conductivity of the brain and nervous tissue, tending later to decrease as exhaustion approaches. The action of drugs, similarly, has definite effects on the electrical conductivity of the various tissues of the body.

One of the more recent applications of electrical conductivity measurements is the adaptation of the method to the study of winter hardiness in plants.[34] Plant tissues which have been exposed to cold were immersed in conductivity water, and it was found that the salts in the plant cells diffused through the injured plasma membranes into the external water, the rate and extent of diffusion being measured by the electrical conductivity of the external water. Table LII shows repre-

TABLE LII

EXOSMOSIS OF ELECTROLYTES AS A MEASURE OF THE COLD INJURY OF WHEAT PLANTS (EXOSMOSIS, 16 HR. AT 0° C., MEASURED BY ELECTRICAL CONDUCTIVITY OF EXTERNAL WATER PHASE)

Wheat Variety	Unhardened	Hardened	
	Oct. 25, Exposed 2 Hr. at −10° C., mhos × 10^6	Nov. 7, Exposed 2 Hr. at −10° C., mhos × 10^6	Dec. 6, Exposed 2 Hr. at −20° C., mhos × 10^6
Minhardi	725	243	338
Wisconsin No. 2	707	309	540
Trumbull	767	527	684

sentative data on three winter wheats in the hardened and unhardened state. The last two columns of the table agree with the agronomic observations of winter hardiness, *i.e.*, Minhardi > Wisconsin No. 2 > Trumbull.

SURFACE CONDUCTANCE.—As already noted in the discussion of electrokinetic phenomena, Briggs found that the electrical conductivity of a colloid gel was not necessarily related to the presence or the concentration of ionized electrolytes. He found that a membrane of pure cellulose immersed in conductivity water acted as a fairly efficient

[34] Dexter, S. T., Tottingham, W. E., and Graber, L. F., Investigations of the Hardiness of Plants by Measurement of Electrical Conductivity, *Plant Physiol.*, 7: 63–78 (1932).

conductor of an electric current, and was able to demonstrate that the conductance was not due to inorganic constituents present in the system. Thus, if a mixture of cellulose fibers in conductivity water was placed in a conductivity cell, and the fibers were allowed to settle below the level of the electrodes, a specific conductivity as low as 4×10^{-6} mho was realized. On shaking the cell so as to bring the cellulose fibers into suspension between the electrodes, a very marked increase in specific conductivity could be obtained, and when the fibers again were allowed to settle, the water between the electrodes regained its original conductivity.

Later Briggs [35] discussed the theory of surface conductance, pointing out that Smoluchowski [36] was incorrect in stating that surface conductance was a function of the ζ-potential. Briggs found surface conductance to be independent of the ζ-potential, as illustrated in Fig. 118, where the solid lines represent ζ-potential curves, the dotted lines, surface conductance curves on the systems. Figure 119, likewise taken from the data of Briggs, shows that there is a definite lyotropic series of ions which influence surface conductance.

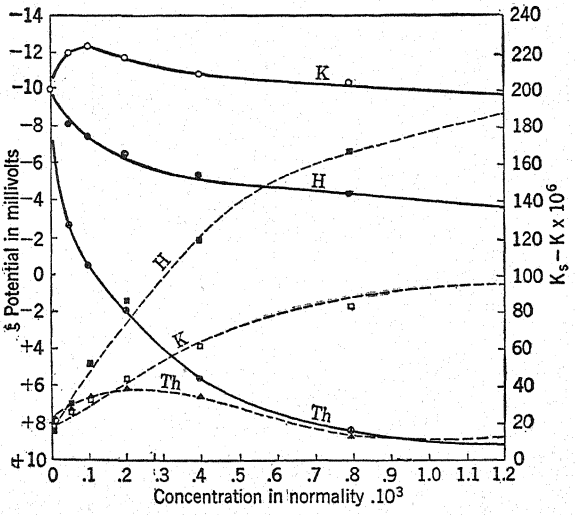


FIG. 118.—Showing that surface conductance is not a function of the electrokinetic potential for a cellulose membrane against salt solutions of varying concentrations. (Data of Briggs.)

Certain systems, such as silica gel, show high surface conductance, whereas other systems, such as flowers of sulfur, Al_2O_3, show relatively slight surface conductance.

Bikerman [37] in a theoretical paper points out that the difficulty

[35] Briggs, D. R., Surface Conductance, Colloid Symposium Monograph, Vol. VI, pp. 41–52, Chemical Catalog Company, New York (1928).

[36] Smoluchowski, M. von, Zur Theorie der elektrischen Kataphorese und der Oberflächenleitung, *Physik. Z.*, 6: 529–531 (1905).

[37] Bikerman, J. J., Ionentheorie der Elektrosmose, der Strömungsströme und der Oberflächenleitfähigkeit, *Z. physikal. Chem.*, Abt. A, 163: 378–394 (1933).

with Smoluchowski's equation lay in the fact that the thickness of the double layer was assumed to be a constant and that the dielectric constant within the double layer was the same as the dielectric constant of the liquid in bulk. Both these assumptions are unjustified. On the basis of the Gouy diffuse layer, Bikerman concludes that surface conductance is a property of the "ion atmosphere" of the surface. Accordingly for any given colloidal material the amount of electricity

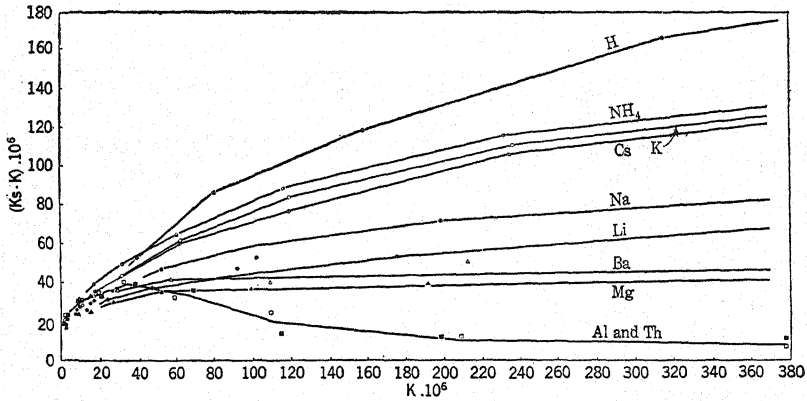


FIG. 119.—Illustrating the valence and lyotropic effect of cations upon the surface conductance of a cellulose membrane. (Data of Briggs.)

which will be conducted along the surfaces will be determined by (*a*) the degree of dispersion, (*b*) the nature of the molecules and ions present in the system, and (*c*) their particular orientation, and probably also (*d*) the affinity of the surface (wettability) for the dispersions medium. There is every indication that the specific conductivity of biological materials is in part due to surface conductance through the lyophilic colloid gel structure, as well as to ionic conductance through the liquid in the cells and intracellular spaces.

CHAPTER XI

THE DONNAN EQUILIBRIUM

As already noted under the discussion of diffusion, two gases separated by a membrane permeable to both will diffuse through the membrane in both directions, so that at equilibrium the mixture will have the same composition on both sides of the membrane. Similarly, if a membrane separates two solutions of different concentration and if the membrane is permeable to both the solute and the solvent, equilibrium will be attained only when the concentration of the liquid on both sides of the membrane is identical. Thus, if a solution of potassium chloride is placed on one side of a permeable collodion membrane and a solution of equivalent concentration of sodium sulfate is placed on the opposite side of the membrane, at equilibrium it would be found that there is an equal concentration of potassium chloride and sodium sulfate on both sides of the membrane, assuming that the membrane is chemically inactive, merely acting as a septum to keep the initial solutions from mixing mechanically. Under such conditions, the osmotic pressure of the solutions in both compartments will be identical.

Donnan[1] notes, however, that very different conditions prevail when the membrane is impermeable to one of the ions. Assuming a compound, NaR, where R represents a colloid micelle, or an ion so large that it is unable to pass through the pores of the diaphragm, and assuming complete dissociation of the NaR into Na^+ and R^-, and further assuming complete dissociation of sodium chloride, and equal volume of liquids on both sides of the membrane, we can represent the hypothetical initial state as:

Na^+	Na^+
R^-	Cl^-
(1)	(2)

[1] Donnan, F. G., Theorie der Membrangleichgewichte und Membranpotentiale bei Vorhandensein von nicht dialysierenden Elektrolyten. Ein Beitrag zur physikalisch-chemischen Physiologie, *Z. Elektrochem.*, 17: 572–581 (1911).

which at equilibrium and constant volume becomes

Na^+	Na^+
R^-	Cl^-
Cl^-	
(1)	(2)

owing to the diffusion of NaCl from compartment (2) through the membrane to compartment (1), the NaR being restrained from diffusing by impermeability of the membrane to the anion.

In this equilibrium the necessary work for the isothermal reversible transference of a molecule of Na^+ from (2) to (1) is just as great as the work which is gained for the corresponding isothermal reversible transference of a molecule of Cl^-. Thus, if we consider the infinitely small isothermal and reversible change of the system in which dn molecules of Na^+ and dn molecules of Cl^- are transferred from (2) to (1), the work gained through this transference (the increase in free energy) is *nil*, and we can therefore write

$$dn \cdot RT \log_e \frac{(Na^+)_2}{(Na^+)_1} = - dn \cdot RT \log_e \frac{(Cl^-)_2}{(Cl^-)_1} \tag{146}$$

Dividing through by $(dn \cdot RT)$, we have the expression,

$$\log_e \frac{(Na^+)_2}{(Na^+)_1} = - \log_e \frac{(Cl^-)_2}{(Cl^-)_1} \tag{147}$$

or

$$\log_e (Na^+)_2 - \log_e (Na^+)_1 = - \log_e (Cl^-)_2 + \log_e (Cl^-)_1 \tag{148}$$

or

$$\log_e (Na^+)_2 + \log_e (Cl^-)_2 = \log_e (Cl^-)_1 + \log_e (Na^+)_1 \tag{149}$$

or

$$(Na^+)_2 \cdot (Cl^-)_2 = (Cl^-)_1 \cdot (Na^+)_1 \tag{150}$$

where the quantity within the parentheses means the molar concentration of the respective ions.

Equation (150) states that *the product of the sodium and chlorine ion concentrations in compartment (2) at equilibrium is equal to the product of the sodium- and chlorine-ion concentrations in compartment (1)*. Since compartment (2) contains only sodium chloride at equilibrium, the chlorine-ion concentration in compartment (2) must be equal to the sodium-ion concentration.

Accordingly,

$$[(Cl^-)_2]^2 = (Na^+)_1 \cdot (Cl^-)_1 \tag{151}$$

Since at equilibrium NaR, as well as NaCl, is present in compartment (1), the concentration of $(Na^+)_1$ will be greater than the concentration of $(Cl^-)_1$. This means that there will be an unequal distribution of ions on the two sides of the membrane at equilibrium, and

accordingly *at equilibrium* the liquid on one side of the membrane may have an appreciably higher osmotic pressure than the liquid on the opposite side of the membrane.[2]

Assuming that x molecules of sodium chloride diffused from compartment (2) to compartment (1), we would have, assuming constant volume, the following concentrations of ions on the two sides of the membrane:

Original State				State of Equilibrium			
Na^+	R^-	Na^+	Cl^-	Na^+	R^- Cl^-	Na^+	Cl^-
C_1	C_1	C_2	C_2	$C_1 + x$	C_1 x	$C_2 - x$	$C_2 - x$
(1)		(2)		(1)		(2)	

where C_1 and C_2 are the original molar ion concentrations in compartments (1) and (2).

Equation (150) affords the following algebraic solution for the quantity of sodium chloride which is transferred from compartment (2) to compartment (1):

$$(C_1 + x) \cdot x = (C_2 - x)^2 \tag{152}$$

or

$$x = \frac{(C_2)^2}{C_1 + 2C_2} \tag{153}$$

and

$$\frac{x}{C_2} = \frac{C_2}{C_1 + 2C_2} \tag{154}$$

or

$$\frac{C_2 - x}{x} = \frac{C_1 + C_2}{C_2} \tag{155}$$

The percentage of sodium chloride which will diffuse from (2) to (1) is accordingly $\frac{x}{C_2} \cdot 100$, and the ratio of the division of sodium chloride between compartment (2) and compartment (1) at equilibrium is given by $\frac{C_2 - x}{x}$.

Tables LIII and LIV show the distribution of sodium chloride at equilibrium under the conditions that we have been discussing, for various initial concentrations of NaR and sodium chloride in com-

[2] The student should remember that all the thermodynamic considerations discussed in this chapter and the generalizations apply to systems *where the volume of the liquid on the two sides of the membrane is kept constant and where only the transference of ions takes place.* Obviously, if the liquid molecules can move so that the volume of the liquid on the two sides of the membrane can change, then it will be impossible to build up an *equilibrium* osmotic pressure which will be greater on one side of the membrane than on the other.

TABLE LIII

DISTRIBUTION OF SODIUM CHLORIDE ON THE TWO SIDES OF A MEMBRANE PERMEABLE TO SODIUM CHLORIDE BUT IMPERMEABLE TO A COLLOIDAL ION (R^-) IN THE SOLUTION ON ONE SIDE OF THE MEMBRANE (CALCULATIONS OF DONNAN)

Original State				State of Equilibrium				
Na^+ C_1	R^- C_1	Na^+ C_2	Cl^- C_2	Na^+ $C_1 + x$	R^- C_1	Cl^- x	Na^+ $C_2 - x$	Cl^- $C_2 - x$
1000	1000	1000	1000	1333	1000	333	666	666
1000	1000	100	100	1008	1000	8.3	92	92
100	100	1000	1000	576	100	476	524	524

TABLE LIV

DISTRIBUTION RATIO OF SODIUM CHLORIDE ON THE TWO SIDES OF A MEMBRANE PERMEABLE TO SODIUM CHLORIDE BUT IMPERMEABLE TO A COLLOIDAL ION (R^-) IN THE SOLUTION ON ONE SIDE OF THE MEMBRANE (CALCULATIONS OF DONNAN)

Original Concentration of NaR in (1) C_1	Original Concentration of NaCl in (2) C_2	Original Ratio of NaR to NaCl $\frac{C_1}{C_2}$	Per Cent NaCl Going from (2) to (1) $\frac{100\ x}{C_2}$	Ratio of NaCl between (2) and (1) $\frac{C_2 - x}{x}$
0.01	1.00	0.01	49.7	1.01
0.10	1.00	0.10	47.6	1.10
1.00	1.00	1.00	33.0	2.00
1.00	0.10	10.00	8.3	11.00
1.00	0.01	100.00	1.0	99.00 (?)

partment (1) and compartment (2). These tables show that the action of non-dialyzable but electrolytically dissociated NaR is very important. Although the membrane is completely permeable to sodium chloride, a high enough concentration of NaR will practically inhibit the diffusion of sodium chloride through the membrane. Conversely, if sodium chloride were added to the compartment containing the non-diffusible ion, the presence of the non-diffusible ion would cause the *excretion* of sodium chloride from this compartment, *such excretion taking place even against an opposing osmotic pressure.* The membrane which is freely permeable to sodium chloride thus shows an apparent differential permeability for the completely dialyzable sodium chloride, this differential permeability being due not to the membrane but to the ionic micelles in the sol on one side.

As Donnan points out, this phenomenon must play a role in physiology. Non-dialyzable anions and cations are present in the cell contents and in the intracellular fluids. The membranes may themselves be completely permeable to molecularly dissolved solutes, but owing to the influence of the ionic micelles, an unequal distribution of ions occurs on the two sides of the membrane.

Donnan further takes up a consideration of an electrolyte having no common ion with the ionic micelle. Considering, in this instance, NaR on one side of the membrane and potassium chloride on the opposite side of the membrane, we have the following, as the original and final states, again assuming constant volume:

Original State		State of Equilibrium	
Na^+	K^+	Na^+	Na^+
R^-	Cl^-	K^+	K^+
(1)	(2)	Cl^-	Cl^-
		R^-	
		(1)	(2)

Here we have three kinds of isothermal reversible changes in the system:

(*a*) The transference of *dn* mole Na^+ from (1) to (2), and the transference of *dn* mole K^+ from (2) to (1).

Accordingly,

$$dn \cdot RT \log_e \frac{(Na^+)_1}{(Na^+)_2} + dn \cdot RT \log_e \frac{(K^+)_2}{(K^+)_1} = 0 \qquad (156)$$

from which by the same algebraic procedure that was used earlier,

$$\frac{(Na^+)_1}{(Na^+)_2} = \frac{(K^+)_1}{(K^+)_2} \qquad (157)$$

(*b*) The transference of *dn* mole Na^+ from (1) to (2), and the transference of *dn* mole Cl^- from (1) to (2).

Accordingly,

$$\frac{(Na^+)_1}{(Na^+)_2} = \frac{(Cl^-)_2}{(Cl^-)_1} \qquad (158)$$

(*c*) The transference of *dn* mole K^+ from (2) to (1), and the transference of *dn* mole Cl^- from (2) to (1).

Accordingly,

$$\frac{(K^+)_1}{(K^+)_2} = \frac{(Cl^-)_2}{(Cl^-)_1} \qquad (159)$$

By means of simultaneous equations we can solve for the quantity of sodium chloride which appears in compartment (2) and for the quantity of potassium chloride which is transferred from compart-

ment (2) to compartment (1), assuming again complete dissociation of the salts and equal and constant volumes of liquid on the two sides of the membrane. The diffusion changes may be represented as follows:

Original State				State of Equilibrium							
Na^+	R^-	K^+	Cl^-	Na^+	K^+	Cl^-	R^-	K^+		Na^+	Cl^-
C_1	C_1	C_2	C_2	$C_1 - z$	x	y	C_1	$C_2 - x$	z		$C_2 - y$
(1)		(2)		(1)				(2)			

where z = the number of Na^+ ions transferred from (1) to (2);
x = the number of ions of K^+ transferred from (2) to (1);
y = the number of ions of Cl^- transferred from (2) to (1).

From equations (157), (158), and (159),

$$\frac{(Na^+)_1}{(Na^+)_2} = \frac{(K^+)_1}{(K^+)_2} = \frac{(Cl^-)_2}{(Cl^-)_1} \tag{160}$$

and by algebraic procedure Donnan finds the following values for x, y, and z.

$$x = \frac{(C_1 + C_2)C_2}{C_1 + 2C_2} \tag{161}$$

$$y = \frac{(C_2)^2}{C_1 + 2C_2} \tag{162}$$

$$z = x - y \tag{163}$$

The above relationships allow us to solve for the actual distribution at equilibrium, as shown in Table LV. Referring to this table, it

TABLE LV

ORIGINAL CONCENTRATIONS AND EQUILIBRIUM CONCENTRATIONS OF A SYSTEM OF NaR AND KCl SEPARATED BY A MEMBRANE IMPERMEABLE TO THE ANION (R^-) BUT FREELY PERMEABLE TO THE OTHER IONS

Original State				State of Equilibrium						
Na^+ C_1	R^- C_1	K^+ C_2	Cl^- C_2	Na^+ $C_1 - z$	K^+ x	R^- C_1	Cl^- y	K^+ $C_2 - x$	Na^+ z	Cl^- $C_2 - y$
1000	1000	1000	1000	666	666	1000	333	333	333	666
1000	1000	100	100	916.7	91.7	1000	8.3	8.3	83.4	91.7
100	100	1000	1000	52	524	100	476	476	48	524

will be noted that there are very great differences in the composition of the equilibrium solution on the two sides of the membrane and that these differences depend very largely upon the initial concentrations. This is more clearly shown in Table LVI.

TABLE LVI

CHANGE IN THE EQUILIBRIUM CONCENTRATION OF IONS ON THE TWO SIDES OF A MEMBRANE WHEN THE MEMBRANE IS IMPERMEABLE TO ONE OF THE IONS ORIGINALLY PRESENT IN COMPARTMENT (C_1)

Ratio of Original Concentration, $C_1 : C_2$	Ratio of Equilibrium Concentration, $C_1 : C_2$
1 : 1	2 : 1
10 : 1	11 : 1
1 : 10	1.1 : 1

It will be noted that, when a relatively large amount of NaR is on one side of the membrane with a relatively small amount of potassium chloride on the other side of the membrane, there is almost complete transference of the K^+ ions from (2) to (1). Thus, a high concentration of NaR in (1) will separate almost completely the K^+ from the Cl^- with which it was originally associated, carrying the K^+ across the membrane and selectively leaving the Cl^- behind. *We have here an excretion of sodium and the selective adsorption of potassium across a membrane, due not to a differential permeability of the membrane but rather to the presence of ionic micelles on one side of the membrane.* It is entirely probable that the selective adsorption of potassium by plants from the soil solution or by marine algae from the sea water is due to a Donnan equilibrium, such as is noted in the above calculations. It may well be that some similar mechanism accounts for the excretion of urea through the tubules of the kidneys, which appear to have a one-sided permeability.

Donnan continues his discussion, taking up the question of the hydrolytic decomposition of salts through the action of a membrane. If NaR is placed on one side of a membrane and pure water is placed on the opposite side, we should have, owing to the presence of hydrogen and hydroxyl ions in the water, the following original and equilibrium conditions.

Original State		State of Equilibrium	
Na^+	H^+	Na^+	Na^+
R^-	OH^-	H^+	OH^-
(1)	(2)	R^- (1)	(2)

Here Donnan shows that

$$\frac{(Na^+)_1}{(Na^+)_2} = \frac{(OH^-)_2}{(OH^-)_1} \qquad (164)$$

Assuming the original and equilibrium states of

Original State			State of Equilibrium				
Na^+	R^-	Pure Water	Na^+	H^+	R^-	Na^+	OH^-
C_1	C_1		$C_1 - x$	x	C_1	x	x
(1)		(2)	(1)			(2)	

the value of x can be determined from the dissociation constant of water.

$$x \cdot (OH^-)_1 = K_w \tag{165}$$

from which Donnan obtains the value of (x) as

$$x = \sqrt[3]{K_w C_1} \tag{166}$$

Table LVII shows Donnan's calculations for the value of x at various concentrations of NaR and the percentage of Na^+ which is transferred across the membrane to form NaOH.

TABLE LVII

HYDROXYL-ION CONCENTRATION (x) OUTSIDE OF A MEMBRANE AT EQUILIBRIUM AND THE PERCENTAGE OF $Na^+ \left(\frac{100\,x}{C_1}\right)$ TRANSFERRED ACROSS THE MEMBRANE FOR VARIOUS ORIGINAL CONCENTRATIONS OF NaR ON ONE SIDE OF THE MEMBRANE AND OF PURE WATER ON THE OTHER SIDE OF THE MEMBRANE

C_1	x	$\frac{100\,x}{C_1}$
		Per Cent
0.01	5.10^{-6}	0.05
0.10	1.10^{-5}	0.01
1.00	2.10^{-5}	0.002

In this case, one would have alkali excreted through the membrane, the liquid on the inside of the membrane becoming more acid. On the other hand, assuming a positively charged ionic micelle, we might very well have the following initial state and equilibrium state:

Original State		State of Equilibrium	
R^+	H^+	R^+	H^+
Cl^-	OH^-	Cl^-	Cl^-
		OH^-	
(1)	(2)	(1)	(2)

In this case we should have *acid excreted* across the membrane.

Donnan points out that *the proper ampholyte can easily give rise by this means alone to a concentration of hydrogen ions in the external liquid as great as that found in the gastric juice.*

Donnan [3-5] has subjected this theory to experimental studies and finds that the theoretical considerations are realized experimentally.

Loeb [6] made a series of elaborate studies of the influence of acids, bases, and salts on protein systems, and, because of the fact that such systems obeyed the Donnan equilibrium, he came to the conclusion that acids and bases, and in some instances salts, combined in stoichiometrical proportion with the proteins, forming definite compounds which could be characterized as "protein chloride," "sodium proteinate," etc. It may eventually be demonstrated that Loeb was correct in assuming a stoichiometrical combination between the protein and the acid or the base. The fact that a definite Donnan equilibrium is set up, however, is no proof of such a relationship, as has already been indicated by Hill,[7] and by Rinde.[8]

The swelling of proteins has been assumed by many investigators to be due to a Donnan equilibrium. Wilson [9] and Loeb and his school have been especially prominent proponents of the Donnan equilibrium. On the other hand, Miss Lloyd [10] finds no evidence for a Donnan equilibrium as influencing the swelling of silk gut in alkaline solutions. Instead the postulate is made that coordinate linkages are opened between the carbonyl group of one peptide chain and the imino group of another and that there is a "binding" of water on the groups so released. The author believes that Miss Lloyd's theory affords the more satisfactory explanation, since under the Donnan equilibrium theory, as developed by Wilson and Loeb, the protein micelle would have to act not only as the non-diffusible ion but also as the membrane which restrained the diffusion of the ions, and in those proteins which are monomolecularly dispersed in solution this seems like a rather im-

[3] Donnan, F. G., and Garner, W. E., Equilibria across a Copper Ferrocyanide and an Amyl Alcohol Membrane, *J. Chem. Soc.*, 115: 1313–1328 (1919).

[4] Donnan, F. G., and Allmand, A. J., Ionic Equilibria across Semipermeable Membranes, *J. Chem. Soc.*, 105: 1941–1963 (1914).

[5] Donnan, F. G., and Harris, A. B., The Osmotic Pressure and Conductivity of Aqueous Solutions of Congo Red, and Reversible Membrane Equilibria, *J. Chem. Soc.*, 99: 1554–1577 (1911).

[6] Loeb, Jacques, Proteins and the Theory of Colloidal Behavior, McGraw-Hill Book Company, New York (1922). (*Cf.* also numerous articles in the *Journal of General Physiology*, Vols. 1–6, inclusive.)

[7] Hill, A. V., The Potential Difference Occurring in a Donnan Equilibrium and the Theory of Colloidal Behavior, *Proc. Roy. Soc., (London)*, (A)102: 705–710 (1923).

[8] Rinde, H., A Method for the Determination of the Adsorption of Ions on Colloidal Particles by Means of Donnan's Membrane Equilibrium Theory, *Phil. Mag.*, (7) 1: 32–50 (1926).

[9] Wilson, J. A., The Chemistry of Leather Manufacture, Second Edition (2 vols.), Chemical Catalog Company, New York (1928–1929).

[10] Lloyd, D. J., and Marriott, R. H., The Swelling of Protein Fibres. II. Silk Gut, *Trans. Faraday. Soc.*, 29: 1228–1240 (1933).

probable hypothesis. Miss Lloyd's viewpoint will be further elaborated when we consider her theories of protein structure.

Although the NaR or the RCl of the hypothetical compounds which are placed on one side of the membrane may represent chemical individuals, non-diffusible anions or cations, respectively, it is equally possible that an adsorption complex, such as we have already postulated in a consideration of the complex theory of colloids, could give rise to a non-diffusible ion and a diffusible ion. Thus, a ferric oxide hydrosol, stabilized by *adsorbed* ferric chloride, would yield an ionic micelle which could be represented by $([Fe_2O_3 \cdot nH_2O]_x\ [FeCl_3]_y)^+$ and Cl^-, or the ionic micelle might acquire a positive charge, owing to the adsorption of a ferric ion. Its composition then would be represented by $(Fe_2O_3 \cdot nH_2O)_x\ Fe^{+++}$. In either instance we would have an ionic micelle for which no stoichiometrical formula could be written. If, however, such a micelle were on one side of the membrane, it would behave as a non-diffusible ion and produce a Donnan equilibrium. Accordingly *a Donnan equilibrium is set up whenever colloidal micelles, carrying either a positive or a negative charge, are concentrated on one side of a membrane, the membrane being impermeable to such micelles but permeable to the ions of an electrolyte on the other side of the membrane.*

An inspection of the preceding tables, showing the unequal distribution of diffusible ions on the two sides of the membrane, probably accounts in a considerable measure for the concentration of electrolytes within plant or animal cells and tissues. Contrary to what one would expect, *the Donnan equilibrium provides a mechanism whereby such cells and tissues may possess a considerably higher osmotic pressure than the liquid which bathes them and at the same time be in equilibrium with the external liquid.*

However, one must not consider that the Donnan equilibrium offers a complete explanation for the adsorption of ions from the soil solution by the root hairs of plants. Even though probably it plays a role, other, as yet unknown, factors are involved. *The cell membrane, at least in certain instances, is more than a mechanical sieve restraining the diffusion of ionic micelles.* Harris and his co-workers [11-15] have

[11] Harris, J. A., Lawrence, J. V., and Lawrence, Z. W., The Chlorid Content of the Leaf Tissue Fluids of Egyptian and Upland Cotton, *J. Agr. Research,* 28: 695–704 (1924).

[12] Harris, J. A., Hoffman, C. T., and Hoffman, W. F., Sulphate Content of the Leaf-Tissue Fluids of Egyptian and Upland Cotton, *J. Agr. Research,* 31: 653–661 (1925).

[13] Harris, J. A., Hoffman, W. F., Sinclair, W. B., Johnson, A. H., and Evans, R. D., The Leaf-Tissue Fluids of Egyptian Cottons, *J. Agr. Research,* 31: 1027–1033 (1925).

[14] Harris, J. A., Hoffman, W. F., and Lawrence, J. V., Differential Absorption of Anions by Varieties of Cotton, *Proc. Soc. Exptl. Biol. Med.,* 22: 350–352 (1925).

[15] Harris, J. A., The Accumulation of Chlorides in the Leaf Tissue Fluids of

shown that the leaf tissue fluids of Egyptian and Upland cotton in the Gila river valley of Arizona differ very markedly in the content of chloride and sulfate ions, the Egyptian type having a chloride content from 28 to 189 per cent higher than the Upland cottons, whereas the Upland cottons have a sulfate content 18 to 28 per cent higher than the Egyptian cottons.

The surprising feature of these investigations is that *the ability of the plant to selectively absorb sulfates and chlorides from the saline soil solution is heritable.* The F_1 crosses between Egyptian and Upland types yielded progeny which were intermediate between the parents in this respect, whereas the ability to selectively absorb either chlorides or sulfates segregated in the F_2 generation.

In these experiments there was no possibility that the results were due to soil heterogeneity. The plants under experiment were alternated in the row and almost invariably showed the higher sulfate or higher chloride content characteristic of the variety. Similarly, the F_1 generation and the F_2 generation were spaced methodically over the fields and still showed in the F_1 intermediate characteristics and in the F_2 the characteristic segregation. The physical mechanism responsible for the selective accumulation of sulfates in the tissue fluids of one form and of a preponderance of chlorides in the tissue fluids of the other form still remains to be worked out.

We have covered in mere outline the basic considerations underlying the Donnan equilibrium. For a further elaboration of the theory and a survey of the applications of the Donnan equilibrium to chemical, physiological, and technological processes, the student is referred to the most excellent review by Bolam.[16]

Egyptian Cotton with the March of the Season, *Proc. Soc. Exptl. Biol. Med.*, 22: 415–417 (1925).

[16] Bolam, T. R., Die Donnan-Gleichgewichte und ihre Anwendung auf chemische, physiologische und technische Prozesse, *Kolloid-Beihefte*, 39: 140–258 (151 refs.) (1934).

II

PROTEINS

"Da die Proteïnstoffe bei allen chemischen Prozessen im lebenden Organismus auf die eine oder andere Weise beteiligt sind, so darf man von der Aufklärung ihrer Struktur und ihrer Metamorphosen die wichtigsten Aufschlüsse für die biologische Chemie erwarten."

EMIL FISCHER (1906)

CHAPTER XII

THE AMINO ACIDS, THE PRIMARY DECOMPOSITION PRODUCTS OF PROTEINS

EARLY INVESTIGATIONS.—With the remarkable development of organic chemistry which began early in the nineteenth century it is not surprising that the attention of the chemist was directed toward a study of the proteins, inasmuch as proteins are a major constituent of every living cell.

The methods which the organic chemist developed, *i.e.*, the elementary analyses for C, H, N, O, and S, were early applied, but it was impossible, owing to the complex nature of the proteins, to differentiate the proteins on the basis of their elementary analysis. Accordingly the early chemists reverted to the other analytical procedures of the organic laboratory. Proteins were subjected to oxidation, using chromic acid, neutral, acid, and alkali solutions of potassium permanganate; to reduction, using tin and hydrochloric acid, hydriodic acid, etc.; to destructive distillation; to fusion with alkalies; to the action of concentrated and fuming nitric acid in attempts to prepare nitro derivatives; to the action of halogens in attempts to prepare characteristic halogen derivatives; to sulfonation; in fact, to all the usual procedures of the organic laboratory.

At the time these studies were carried out nothing was known of the chemical nature of the proteins, and accordingly it is not surprising that little information was gained from the application of such technic. Now that we have an insight into the chemical nature of the proteins, and accordingly have some idea as to what products might be formed by the actions of the various reagents, it would be highly desirable to repeat many of these older studies in the light of modern information and modern technic. To some extent this is being done at the present time.

Johnson [1] successfully nitrated various proteins, and Johnson [2] and Pictet [3] subjected proteins to destructive distillation, securing valuable

[1] Johnson, T. B., *et al.*, Studies on Nitrated Proteins, I–V, *J. Am. Chem. Soc.*, 37: 1863–1884, 2164–2170, 2170–2178, 2598–2603, (1915); 38: 1392–1398 (1916).

[2] Johnson, T. B., and Daschavsky, P. G., Researches on Proteins. VI. Destructive Distillation of Fibroin, *J. Am. Chem. Soc.*, 41: 1147–1149 (1919).

[3] Pictet, A., and Cramer, M., Sur la distillation de l'ovalbumine sous pression réduite, *Helv. Chim. Acta.*, 2: 188–195 (1919).

data. Similarly, Vandevelde [4] studied the halogen derivatives of the proteins.

In 1848, Guckelberger, in Liebig's laboratory, oxidized egg white, casein, fibrin, and gelatin with potassium permanganate, and potassium bichromate and sulfuric acid. Among the products obtained he identified formic, acetic, propionic, valeric, caproic, and benzoic acids, as well as benzaldehyde and ammonia. However, he was unable to correlate these findings with any rational structure of the protein molecule.

Bernert, using potassium permanganate in alkaline solution, obtained the same list, and, in addition, pyrrol and two amino acids, lysine and histidine. Kutscher obtained guanidine, butyric acid and succinic acid. Lossen had earlier obtained guanidine from proteins by oxidation. Other workers, using hydrogen peroxide in acid solution with ferrous sulfate as a catalyst, obtained acetone and isovaleric aldehyde.

Hlasiwetz and Habermann treated egg albumin with free bromine in an autoclave, and from 100 grams of egg albumin obtained 29.9 grams of bromoform, 22 grams of bromacetic acid, 12 grams of oxalic acid, 23.8 grams of aspartic acid, 22.6 grams of leucine, and 1.5 grams of bromanil (tetrabrom *p*-benzoquinone).

Mann [5] discussed some of these earlier investigations at considerable length. Suffice it to say that while all these studies and many other studies, similar to those which have been noted, showed the great complexity of the protein molecule, they did not contribute much in the way of establishing the essential chemical nature of the proteins or the structure of the protein molecule.

THE AMINO ACIDS AS DECOMPOSITION PRODUCTS OF PROTEINS.—We now know the protein molecule to be made up primarily of amino acids linked together through the amino and carboxyl groups or through other reactive groups present in the amino acids concerned. The real clue to the structure of the proteins came from investigations where hydrolytic methods were employed, hydrolyzing the protein with either (*a*) acids, (*b*) enzymes, or (*c*) alkalies. Vickery and Schmidt [6] have monographed the history of the discovery of the amino acids up to 1931, and the student is referred to their paper for literature citations.

Apparently Braconnot was the first investigator to use acid hy-

[4] Vandevelde, A. J. J., Contribution à l'étude des protéines halogénées. I–V, *Rec. trav. chim.*, 43: 158–162, 326–328, 702–706 (1924); 44: 224–228, 900–906 (1925); Halogenated Proteins, VI–VIII, *Rec. trav. chim.*, 45: 825–829 (1926); 46: 133–136, 590–593 (1927).

[5] Mann, Gustav, Chemistry of the Proteids, Macmillan and Company, London (1906).

[6] Vickery, H. B., and Schmidt, C. L. A., The History of the Discovery of the Amino Acids, *Chem. Rev.*, 9: 169–318 (1931) (415 refs.).

drolysis. In 1820, he boiled both gelatin and meat with dilute sulfuric acid and identified glycine from both proteins. This was the first instance in which an amino acid was shown to be a primary decomposition product of proteins.

Proust had earlier isolated leucine from cheese and had called it "oxide caséeux." This was the first amino acid to be discovered, but the fact that it was present in crystalline form in the holes in the cheese did not prove that it was a primary decomposition product of the protein.

Braconnot's method of acid hydrolysis was not generally recognized as affording a means by which primary protein decomposition products could be obtained, and no further amino acids were discovered until Liebig, in 1846, isolated a crystalline compound from casein which had been hydrolyzed by strong alkali. This compound was later identified as tyrosine. The next amino acid to be discovered was serine, isolated by Cramer by hydrolyzing silk with sulfuric acid.

Kuhne, in 1867, introduced a new method for the study of proteins, *i.e., digestion with tryptic enzymes*, and identified tyrosine and leucine as primary decomposition products. Here again, however, the fact that tryptic digestion afforded a new technic for the study of proteins was not generally recognized.

At about this time, Ritthausen [7] began his extensive series of investigations of the vegetable proteins, and in 1868 added aspartic acid and glutamic acid to the list of known amino acids. These acids were isolated from vegetable proteins by acid hydrolysis. Schützenberger and Bourgeois isolated alanine from silk by hydrolysis with barium hydroxide, and its presence was later confirmed by Weyl, using acid hydrolysis. Schulze and Barbieri isolated phenylalanine from the sap of germinated seeds of *Lupinus luteus*, and, in 1889, Drechsel identified lysine as a constituent of proteins.

Thus, in the period from 1800 to 1890, only nine amino acids were isolated and identified. During the decade 1890 to 1900, four additional amino acids were isolated. In the decade 1900 to 1910, the discovery of eight amino acids was announced, five of which, tryptophane, proline, oxyproline, isoleucine, and valine, are of rather common occurrence. Of the other three, one (β-alanine) has been confirmed but has not as yet been shown to be present in unaltered proteins. The remaining two are of questionable occurrence.

In the decade 1910–1920, seven new amino acids were added to the list. Since 1920 eight additional amino acids have been isolated from biological sources, and there is every indication that additional amino acids remain to be discovered in biological materials.

[7] Ritthausen, H., Die Eiweisskörper der Getreidearten, Hülsenfrüchte und Olsamen, Max Cohen und Sohn, Bonn (1872).

Table LVIII lists the various amino acids, the year of their discovery, their common names, and their scientific names, with the names of the persons making the announcement of discovery. Not all the amino acids listed in Table LVIII have been isolated from proteins, but they have all been isolated from some biological material. Some have been isolated from plant saps or are found in aqueous extracts from plant or animal tissues. A few, such as ornithine, appear in combination with other compounds in animal or plant products. Others may be formed from other amino acids by enzyme action, *e.g.*, ornithine from arginine, and canaline from canavanine. It seems legitimate to list these amino acids in the table, however, for our methods of protein analysis are so inadequate that it may well be that they are primary protein constituents but still remain to be identified as such. Probably no protein has been so extensively investigated as casein, and the inadequacy of our present methods of protein research are strikingly illustrated by the fact that Dakin,[8] in 1918, isolated more than 10 per cent of β-hydroxyglutamic acid from casein. The amino acids which have been actually isolated from proteins in very few instances total more than 70 per cent of the original protein, and accordingly there is every reason for believing that there are a number of amino acids as yet unknown.

Perhaps two of the recently discovered amino acids deserve special mention because they possess rather unusual organic structures. In 1929, Kitagawa and Tomiyama[9] isolated canavanine from the jack bean and in subsequent papers suggested that the properties of the compound were such as to indicate that it possessed the structure, $NH_2—CH(NH)—NH—O—CH_2—CH_2—CH(NH_2)—COOH$. It is thus a guanidine derivative of an α-amino-γ-hydroxy acid in which the linkage between the guanidine group and the acid is through an oxygen, or it may be considered as a derivative of hydroxylamine, for, when it is acted upon by alkalies, urea is split off and canaline is formed, possessing the unusual structure of an amino group linked in the γ position through an oxygen to an amino acid. The other unusual amino acid is djenkolic acid found by van Veen and Hijman[10] in the djenkol nut. This compound has the structure which would result from the condensation of two molecules of cysteine with one molecule of formaldehyde and the splitting off of water from the —SH groups and the oxygen of the formaldehyde. The structure has been confirmed by

[8] Dakin, H. D., On Amino-Acids, *Biochem. J.*, 12: 290–317 (1918).

[9] Kitagawa, M., and Tomiyama, T., A New Amino-Compound in the Jack Bean and a Corresponding New Ferment, *J. Biochem. (Tokyo)*, 11: 265–271 (1929).

[10] van Veen, A. G., and Hijman, A. J., Het giftige bestanddeel van de djengkol, *Geneeskund. Tijdschr. Nederland.-Indië*, 73: 991–1001 (1933); Die Djenkolsäure, eine neue schwelfelhaltige Aminosäure, *Rec. trav. chim.*, 54: 493–501 (1935).

TABLE LVIII

THE NATURALLY OCCURRING AMINO ACIDS

(The existence of a few of these amino acids is doubtful, as noted by "?")

Year	Common Name and Scientific Name (When Known)	Discoverer
1818	Leucine, β-iso-propyl-α-amino-propionic acid	Proust
1820	Glycine or glycocoll, α-amino-acetic acid	Braconnot
1846	Tyrosine, β-(*p*.-hydroxyphenyl) α-amino-propionic acid	Liebig
1865	Serine, β-hydroxy-α-amino-propionic acid	Cramer
1868	Aspartic acid, α-amino-succinic acid	Ritthausen
1868	Glutamic acid, α-amino-glutaric acid	Ritthausen
1875	Alanine, α-amino-propionic acid	Schützenberger and Bourgeois
1877	Ornithine, α-δ-diamino-valerianic acid	Jaffe
1883	Phenylalanine, β-phenyl-α-amino-propionic acid)	Schulze
1889	Lysine, α-ε-di-amino-caproic acid	Drechsel
1895	Arginine, α-amino-δ-guanidino valerianic acid	Hedin
1896	Histidine, β-imidazole-α-amino-propionic acid	Kossel
1896	Iodo-gorgonic acid, 3.5-di-iodo tyrosine	Drechsel
1899	Cystine, β-β′-di-thio-di-(α-amino propionic acid)	Mörner
1901	Tryptophane, β-indol-α-amino-propionic acid	Hopkins and Cole
1901	Proline, pyrrolidine-α-carboxylic acid	Fischer
1901	Cysteine, α-amino-β-thio-lactic acid	Embden
1902	Hydroxy-proline, hydroxy-pyrrolidine-α-carboxylic acid	Fischer
1904	Caseanic acid, $C_{12}H_{24}N_2O_5$ (?)	Skraup
1904 *	Diamino-tri-hydroxy-dodecanoic acid, $C_{12}H_{26}N_2O_5$(?)	Fischer and Abderhalden
1927 *	Dodecan-diamino-di-carboxylic acid, $C_{12}H_{24}N_2O_4 \cdot H_2O$ (?)	Fränkel and Friedmann
1905	Iso-leucine, β-methyl-β-ethyl-α-amino-propionic acid	Winterstein
1906	Valine, α-amino-iso-valerianic acid	Fischer
1907 *	Hydroxy-tryptophane [position of (—OH) uncertain (?)]	Abderhalden and Kempe
1924 *	β-Bz.-hydroxy-Pr.-dihydro-indolyl-alanine (?)	Abderhalden and Sickel
1908	β-alanine, β-amino-propionic acid	Engeland
1913	Nor-leucine, α-amino *n*. caproic acid	Abderhalden and Weyl
1913	α-amino *n*. butyric acid	Foreman
1913	Dopa, 3.4-di-hydroxy-phenylalanine	Guggenheim
1913	3.5-dibrom-tyrosine	Mörner
1914	Citrulline, α-amino-δ-carbamido-valerianic acid	Koga and Odake (Wada, 1930)
1918	β-hydroxy-glutamic acid	Dakin
1919	Thyroxine, β-(3.5.3′5′-tetra-iodio-4′-hydroxy-di-phenyl-ether)-α-amino-propionic acid	Kendall
1922	Methionine, γ-methylthiol-α-amino-butyric acid	Mueller
1925	Hydroxy-lysine	Schryver
1925 *	$C_4H_{11}O_3N$ (?)	Gortner and Hoffman
1926 *	$C_4H_9O_3N$, hydroxy-amino-butyric acid	Schryver and Buston
1935 *	Threonine, β-hydroxy-α-amino-butyric acid	Rose
1926	Hydroxy-valine	Schryver and Buston
1929	Canavanine, α-amino-γ-hydroxy-guanidino-butyric acid	Kitagawa and Tomiyama
1930	Norvaline, α-amino-*n*-valerianic acid	Abderhalden and Bahn
1932	Canaline, α-amino-γ-(*o*)hydroxylamino-butyric acid	Kitagawa and Yamada
1933	Djenkolic acid, *l*-cysteine-thiolformacetal	van Veen and Hijman

* The compounds within the bracket are probably identical.

synthesis, and on hydrolysis djenkolic acid breaks down into cysteine and formaldehyde.

THE DEVELOPMENT OF THE PRESENT VIEW OF THE NATURE OF PROTEINS.—As already indicated, amino acids are the primary decomposition products of proteins. This was established by the work of Emil Fischer who contributed so extensively to this field during the period from 1889 to 1918. He was preeminent not alone in the field of proteins but likewise in the study of carbohydrates, the purine and pyrimidine derivatives, and the tannins. Probably no one person has influenced the fields of biochemistry and organic chemistry more than Emil Fischer.

His work began on the proteins at a time when nine monoamino acids and four diamino acids were known. Eight of the monoamino acids had previously been synthesized and their structures thus definitely proved. Serine and the diamino acids had not been synthesized. It is an axiom that, until an organic compound has been synthesized by known chemical reactions, its structure is still uncertain. Fischer,[11] in 1906, presented an outline of the plan which he developed for the study of the proteins. The first item of the outline was these studies of syntheses.

As a second field, he studied amino acid derivatives, including the preparation and study of the esters and acetyl derivatives, the phenylisocyanates, etc., with a view to finding chemical derivatives which would permit of the separation of mixtures of amino acids. He then proceeded to the experimental separation of mixtures of amino acids, such as occur by the hydrolysis of unaltered proteins.

Simultaneously with studies on the separation of the amino acids, he attempted to recombine two or more amino acids into compounds which he designated by the name of "polypeptides" and studied the chemical and physical properties of such derivatives, particularly in their relation to protein structure. These polypeptides were subjected to hydrolysis, were tested as to their behavior toward enzyme action, and in various ways contributed much to our knowledge of the chemistry of the proteins.

He further investigated the proteins themselves. He was interested in the problem of protein structure, in a critical study of the various methods for protein hydrolysis, conducted extensive series of analyses of various proteins, and studied in a general way the protein molecule as an entity. Remarkable as it may seem, he completed very satisfactorily and in a most extraordinary manner the entire program which he planned, and today we are indebted to his technics for much of the available information on the organic chemistry of the protein molecule.

[11] Fischer, E., Untersuchungen über Aminosäuren, Polypeptide und Proteine, *Ber.*, 39: 530–610 (1906).

Four other workers may be credited with laying the foundation of the modern viewpoint. Kossel, working during the period of 1895 to 1915, made major contributions to protein chemistry in his study of the diamino acids, including the Kossel method for their quantitative separation and identification (*vide infra*). He contributed much to the general field of protein chemistry but was particularly interested in the histones and the protamines, the basic proteins characterized by a high content of diamino acids.

Abderhalden received his basic training in the field of proteins under the guidance of Emil Fischer and is probably the most prolific research worker in the field. Since 1904 he has published literally hundreds of papers dealing with one or another phase of protein chemistry, in addition to many papers in other fields. No consideration of workers in the field of proteins would be complete without the acknowledgment of the advances which have been made by Abderhalden and his students.

As already noted, Ritthausen early began an investigation of the vegetable proteins. Owing to the lack of organized technic and any definite knowledge of protein structure, Ritthausen's contributions, though important, were inadequate.

Thomas B. Osborne began work with vegetable proteins about 1895 and from that time until his death in 1929 was recognized as the outstanding authority in this field of protein research. It is to Osborne that we owe many improvements in the methods of protein analysis and much of our information in regard to the methods of isolation and purification of the vegetable proteins.

During the nineteenth century, organic chemistry dominated chemical science. Toward the end of the nineteenth century, however, physical chemistry began more and more to assume a supremacy. It is not surprising, therefore, that many of the more important recent contributions in the field of protein study should be characterized by the application of physicochemical methods. In 1909, S. P. L. Sörensen demonstrated the importance of hydrogen-ion concentration to biological and biochemical reactions, and, in 1917, he published a series of papers [12] in which the most exact physicochemical technics, including the influence of hydrogen-ion concentration, were applied to the problems of protein behavior. In the intervening years since that time there have come from his laboratory numerous contributions dealing with the physicochemical properties of protein systems, and although many other workers in other laboratories are now utilizing physicochemical technics, it seems fitting to include Sörensen in the list of those workers in the field of proteins who have given us new tools and new concepts.

[12] Sörensen, S. P. L., Studies on Proteins, *Compt. rend. Lab. Carlsberg*, Vol. 12 (1917).

THE AMINO ACIDS AS ZWITTERIONS.—Apparently the first suggestion that amphoteric compounds (compounds which contain both acidic and basic groups) exist in a special state was made by Adams [13] in 1916. He points out that aminoacetic acid exists almost exclusively as the inner salt, $^{+}NH_3—CH_2—CO_2^{-}$, with probably less than one part per million of the true amino acid, $NH_2—CH_2—COOH$. Bjerrum,[14] in a more complete discussion of the constitution of ampholytes, confirmed and extended Adams' theory and pointed out that all the amino acids exist in the salt-like double ions, $^{+}NH_3RCO_2^{-}$, and that therefore they are not true amino acids but rather internal ammonium salts. Bjerrum coined the name *zwitterions* for this particular type of substances.

In 1930, Harris [15] extended the zwitterion theory, and later investigations of C. L. A. Schmidt, E. J. Cohn, and others have firmly established it. The zwitterion theory has materially changed our conceptions of the relationships regarding the dissociation constants of the amino acids. We have already seen [equations (25) and (26)] that the dissociation constants of acids and bases are the product of the concentration of the anion and the cation divided by the concentration of the undissociated acid. These dissociation constants have been expressed by K_a and K_b. Bjerrum pointed out that in the case of the amino acids these constants were not true dissociation constants but rather hydrolysis constants arising from the hydrolysis of the internal salt. He suggested that the real dissociation constants of the amino acids could be expressed by K_A and K_B where

$$K_A = \frac{K_w}{K_b} \tag{167}$$

and

$$K_B = \frac{K_w}{K_a} \tag{168}$$

Bjerrum points out that the inner-salt nature of amino acids is abundantly proved by many of the properties of the solutions, which are essentially neutral in reaction. The amino acids, in general, are insoluble in organic solvents, are very soluble in water, are often more

[13] Adams, E. C., Relations between the Constants of Dibasic Acids and of Amphoteric Electrolytes, *J. Am. Chem. Soc.*, 38: 1503–1510 (1916).

[14] Bjerrum, N., Die Konstitution der Ampholyte, besonders der Aminosäuren, und ihre Dissoziationskonstanten, *Z. physik. Chem.*, 104: 147–173 (1923).

[15] Harris, L. J., Zwitterions I. Proof of the Zwitterion Constitution of the Amino-Acid Molecule, *Biochem. J.*, 24: 1080–1085 (1930); (with T. W. Birch), II. Amino-Acids, Polypeptides, etc., and Proteins as Zwitterions, with Instances of Non-Zwitterion Ampholytes, *Biochem. J.*, 24: 1086–1097 (1930); (with T. W. Birch), A Redetermination of the Titration Dissociation Constants of Arginine and Histidine with a Demonstration of the Zwitterion Constitution of These Molecules, *Biochem, J.*, 24: 564–575 (1930).

soluble in salt solutions than in pure water, and exhibit many other properties characteristic of true salts.

The zwitterion theory changes decidedly the viewpoint on the reactions of amino acids with acids and bases. Thus, in the older viewpoint the reaction of glycine with hydrochloric acid would have been written

$$NH_2—CH_2—COOH + HCl \rightleftarrows {}^+NH_3—CH_2—COOH + Cl^-$$

whereas under the zwitterion theory the reaction would be expressed

$$^+NH_3—CH_2—COO^- + HCl \rightleftarrows {}^+NH_3-CH_2-COOH + Cl^-,$$

and in the case of bases, the reaction

$$NH_2—CH_2—COOH + NaOH \rightleftarrows NH_2—CH_2—COO^- + Na^+$$

would be altered to

$$^+NH_3—CH_2—COO^- + NaOH \rightleftarrows NH_2—CH_2—COO^- + Na^+.$$

It will be noted that the net result so far as the nature of the final product is concerned is the same but that there are fundamental differences in the mechanism of the reactions. Under the older theories the acids reacted with the amino group to form an ammonium salt. Under the zwitterion theory the acid reacts to set free, from a salt, the carboxyl group of a weak acid. Similarly under the old viewpoint the strong base reacted with the acid group to form a salt, whereas under the zwitterion theory the strong base liberates the basic group of the weaker base in the salt in the same way that sodium hydroxide liberates ammonia from ammonium acetate. *Fundamentally, therefore, acids react with the acidic groups of amino acids and proteins, and bases react with the basic groups of amino acids and proteins.*

Kirk and Schmidt [16] have summarized the literature on the apparent dissociation constants of the amino acids and discuss the theories involved. In Table LIX are given those data for the amino acids which they regard as of greatest validity.

THE ISOELECTRIC POINT OF AMINO ACIDS AND PROTEINS.—We have seen that in the presence of strong acids the amino acid residue exists largely as a cation and in the presence of strong bases it exists as an anion. In pure water, cations, anions, and an undissociated residue are all present with the undissociated residue in great excess. Accordingly there must be some hydrogen-ion concentration at which the undissociated residue has a maximum concentration and at which the sum of the anions plus the cations is minimal. This hydrogen-ion concentration has been defined as the isoelectric point and in terms of elec-

[16] Kirk, P. L., and Schmidt, C. L. A., The Apparent Dissociation Constants of the Amino Acids and Certain Related Substances: A Summary of the Data, *Univ. Calif. Pub. Physiol.*, 7: 57–69 (1929).

TABLE LIX

APPARENT DISSOCIATION CONSTANTS OF THE AMINO ACIDS

Compound	Temperature, ° C.	K_a	K_b
Alanine	25	1.9×10^{-10}	2.3×10^{-12}
β-Alanine	25	6.5×10^{-11}	4.0×10^{-11}
Arginine	25	2.0×10^{-10}	(1) 2.0×10^{-6}
			(2) 1.5×10^{-12}
Aspartic acid	25	(1) 2.0×10^{-4}	1.0×10^{-12}
		(2) 2.0×10^{-10}	
Cysteine	30	(1) *ca.* 7 $\times 10^{-9}$	*ca.* 8 $\times 10^{-1}$
		(2) *ca.* 5 $\times 10^{-11}$	
Cystine	30	(1) 3.3×10^{-8}	(1) 5 $\times 10^{-13}$
		(2) 9.6×10^{-10}	(2) $< 1.5 \times 10^{-13}$
Glutamic acid	25	(1) 6.0×10^{-5}	1.4×10^{-12}
		(2) 2.5×10^{-10}	
Glycine	25	1.8×10^{-10}	2.6×10^{-12}
Histidine	25	3.9×10^{-10}	(1) 1.2×10^{-8}
			(2) 2.9×10^{-13}
β-Hydroxyglutamic acid	25	(1) 5.8×10^{-5}	2.1×10^{-12}
		(2) 2.8×10^{-10}	
Isoleucine	25	2.1×10^{-10}	2.3×10^{-12}
Leucine	25	2.5×10^{-10}	2.3×10^{-12}
Lysine	25	3.0×10^{-11}	(1) 2.0×10^{-5}
	25		(2) 1.0×10^{-12}
Norleucine	25	1.7×10^{-10}	2.5×10^{-12}
Norvaline	25	1.9×10^{-10}	2.3×10^{-12}
Ornithine	25	1.7×10^{-11}	(1) 4.5×10^{-6}
			(2) 8.7×10^{-13}
Oxyproline	25	1.9×10^{-10}	8.3×10^{-13}
Phenylalanine	25	7.5×10^{-10}	4.0×10^{-13}
Proline	25	2.5×10^{-11}	1.0×10^{-12}
Serine	25	7.1×10^{-10}	1.6×10^{-12}
Tryptophane	25	4.1×10^{-10}	2.2×10^{-12}
Tyrosine	25	(1) 7.0×10^{-10}	1.7×10^{-12}
		(2) 7.0×10^{-11}	
Valine	25	2.3×10^{-10}	2.0×10^{-12}

trical transport may be defined as *that hydrogen-ion concentration at which there will be a tendency for as many cations to migrate toward the cathode as there are anions migrating toward the anode.* This point will be reached when

$$-\frac{K_a}{[H^+]^2} + \frac{K_b}{K_w} = 0 \qquad (169)$$

or

$$[H^+]_{I.P.} = \sqrt{\frac{K_a}{K_b} K_w} \qquad (170)$$

where $[H^+]_{I.P.}$ = the hydrogen-ion concentration at which the ampholyte is isoelectric.

This definition of the isoelectric point was originally proposed by Michaelis [17] and has been accepted and somewhat expanded by Eckweiler, Noyes, and Falk,[18] and by Levene and Simms.[19] Levene and Simms point out that, although equation (170) was originally developed for simple monobasic and monoacidic ampholytes, it has a wider application and can be applied to complex ampholytes, such as proteins, by taking into consideration the *strongest acid group* and the *strongest basic group* of the complex ampholyte. For a complex ampholyte the isoelectric point would be defined as:

$$[H^+]_{I.P.} = \sqrt{\left(\frac{K_{a1} + K_{a2} + K_{a3} \ldots + K_{an}}{K_{b1} + K_{b2} + K_{b3} \ldots + K_{bn}}\right) K_w} \qquad (171)$$

or

$$[H^+]_{I.P.} = \sqrt{\frac{\Sigma K_a}{\Sigma K_b} K_w} \qquad (172)$$

It is obvious that, if the second, third, etc., dissociation constants contribute only small amounts to the numerator and the denominator of equation (171), then equation (170), where only the primary dissociations are considered, is approximated.

In this consideration of the isoelectric point we have used the "apparent dissociation constants" and considered the ampholyte to be isoelectric at that hydrogen-ion concentration where it is ionized equally as an acid and as a base and where the isoelectric point may be considered as the point of *minimum* dissociation. As a matter of fact, the zwitterion theory leads to an exactly opposite viewpoint and states that *the isoelectric point is at that hydrogen-ion concentration where the ampholyte exists to the maximum degree as a zwitterion, and where the positive charges on the zwitterion exactly balance the negative charges on the zwitterion, and where there are a minimum of unionized potentially acidic or basic groups.* It may seem somewhat of a paradox that equations (170) and (172) are valid under either consideration and that the isoelectric point calculated by these equations is actually the true isoelectric point of the ampholyte. It should be remembered, however, that the apparent dissociation constants, K_a and K_b, are in reality hydrolysis constants which explains why equations (170) and (172) are valid when the ampholyte is considered as a zwitterion.

[17] Michaelis, L., Die Wasserstoffionenkonzentration, Julius Springer, Berlin (1914).

[18] Eckweiler, H., Noyes, Helen M., and Falk, K. G., The Amphoteric Properties of Some Amino-Acids and Peptides, *J. Gen. Physiol.*, 3: 291–308 (1921).

[19] Levene, P. A., and Simms, H. S., Calculation of Isoelectric Points, *J. Biol. Chem.*, 55: 801–813 (1923).

THE REACTIVE GROUPS IN PROTEINS.—In all his studies on the proteins Fischer emphasized the reactions which take place between a primary amino group and the carboxyl group of an organic acid. In his synthesis of the polypeptides, he formed the peptide linkage

$$R—NH_2 + R'—COOH = R—NH—COR' + H_2O$$

and it was definitely proved to occur in the unaltered proteins. Probably because Fischer emphasized this particular linkage, attention has been focused by research workers almost entirely upon the amino and the carboxyl groups of the proteins, in an attempt to explain protein structure and protein reactions. There is a certain amount of definite evidence, however, that even though the amino groups and carboxyl groups are of primary importance, groups and linkages other than the peptide linkage may and probably do occur in the unaltered proteins. A consideration of the reactive groups present in the various amino acids should indicate to any organic chemist the possibility of other reactions and other linkages. The following groups are present in at least one of the amino acids noted in Table LVIII:

1. Primary amino group ($—NH_2$).
2. Carboxyl group (—COOH), especially in the dicarboxylic acids.
3. Aliphatic alcohol group (—OH).
4. Phenolic group (—OH), (aromatic alcohols).
5. Alcohol group intermediate between the aliphatic (—OH) and the aromatic (—OH), as the (—OH) in oxyproline.
6. Imino group (=NH).
7. Acid amide group ($—CO—NH_2$).
8. Sulfhydryl group (—SH) in cysteine, or the disulfide group (—S—S—) of cystine.
9. α-Hydrogen of tryptophane.
10. The guanidine nucleus.

Several papers in the literature indicate the presence of methoxy ($—OCH_3$) and N-methyl groups in proteins. However, the discovery of methionine which contains an $—S—CH_3$ group has apparently accounted [20] for all the methyl groups reported by the earlier workers.

That the imino group of proline does enter into combination is shown by the presence of polypeptides such as glycyl-*l*-proline where the carboxyl group of glycine is united to the imino group of proline. Bergmann [21] states that more than 25 per cent of the "peptide" link-

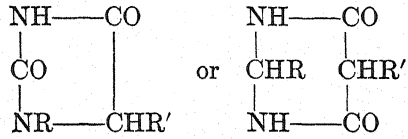
[20] Baernstein, H. D., The Determination of Methionine in Proteins, *J. Biol. Chem.*, 97: 663–668 (1932).

[21] Bergmann, M., Complex Salts of Amino Acids and Peptides. II. Determination of *l*-proline with the Aid of Rhodanilic Acid. The Structure of Gelatin, *J. Biol. Chem.*, 110: 471–479 (1935).

ages in gelatin and collagen are of this type and that this linkage is split by a specific proteolytic enzyme [22] which occurs in erepsin but not in pancreatic juice.

Theoretically the ether linkage is a possibility. The presence of such a linkage, except in the case of thyroxine, still remains to be proved, although the —NH—O—CH_2— linkage in canavanine may be looked upon as a pseudo ether. The —OH groups of the hydroxy amino acids do play an important role in protein structure, since, when they are esterified with phosphoric acid in the diester type, the phosphoric acid forms a bridge between two polypeptide chains. Linkages of this sort are all in the phosphoproteins, *e.g.*, casein where hydroxy-amino acids, such as serine, are involved.

Dunn [23] points out that there is a liberation of carbon dioxide during the acid hydrolysis of a protein, amounting in the case of casein to 0.71 per cent of the weight of the casein. He suggests that the sources of this carbon dioxide may be uramino acids, hydantoins, or cyclic diacipiperazines, possibly in such structures as:



Ssadikow [24] had earlier observed the formation of carbon dioxide when proteins were hydrolyzed in an autoclave but assumed that it was due to oxidation processes inasmuch as no oxygen remained in the residual gases in the autoclave. Incidentally his studies yielded another observation which still remains to be explained. In a second paper [25] he observed that, when proteins were hydrolyzed in an autoclave in a nitrogen atmosphere, the nitrogen was in some manner "fixed" and disappeared from the gas phase.

THE SYNTHESIS OF AMINO ACIDS.—Six general types of reactions have been utilized for the synthesis of α-amino acids.

1. The synthesis of an α-amino acid from an *α-halogen acid* and ammonia:

$$R—CH(Cl)—COOH + NH_3 = R—CH(NH_2)—COOH + HCl$$

[22] Bergmann, M., and Fruton, J. S., A New Type of Enzyme in the Intestinal Tract, *Science*, 83: 306 (1936).

[23] Dunn, M. S., The Liberation of Carbon Dioxide, Ammonia, and Amino Nitrogen from Casein by Acid Hydrolysis, *J. Am. Chem. Soc.*, 47: 2564–2568 (1925).

[24] Ssadikow, W. S., Über die Kohlensäurebildung bei Spaltung der Eiweissstoffe im Autoklaven, *Biochem. Z.*, 143: 492–495 (1923).

[25] Ssadikow, W. S., Über Autoklaven-Spaltungen in einer Stickstoffatmosphäre, *Biochem. Z.*, 143: 496–503 (1923).

This reaction, as a rule, works smoothly, the only difficulty being the securing of the proper halogen acid.

2. The synthesis from an *aldehyde* having one less carbon than the desired amino acid, by the addition of hydrocyanic acid and ammonia, with the subsequent saponification of the nitrile:

$$R{-}CHO + HCN = R{-}CH(OH)CN$$

$$R{-}CH(OH)CN + NH_3 = R{-}CH(NH_2)CN + H_2O$$

$$R{-}CH(NH_2)CN + 2H_2O = R{-}CH(NH_2){-}COOH + NH_3$$

3. The synthesis from *malonic ester* through the halogen compound:

$$CH_2(COOEt)_2 + Na = Na{-}CH(COOEt)_2 + H_2$$

$$Na{-}CH(COOEt)_2 + C_6H_5CH_2Cl = C_6H_5CH_2{-}CH(COOEt)_2 + NaCl$$

$$C_6H_5CH_2{-}CH(COOEt)_2 + Br_2 = C_6H_5CH_2{-}C(Br)(COOEt)_2 + HBr$$

$$C_6H_5CH_2{-}C(Br)(COOEt)_2 + NH_3 = C_6H_5CH_2{-}C(NH_2)(COOEt)_2 + HBr$$

$$C_6H_5CH_2{-}C(NH_2)(COOEt)_2 \xrightarrow[\text{and heat}]{\text{saponify}} =$$

$$C_6H_5{-}CH_2{-}CH(NH_2){-}COOH + 2C_2H_5OH + CO_2$$

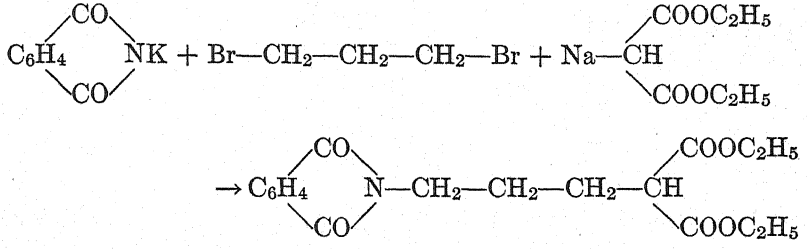
(*i*-phenylalanine)

The above series of reactions was carried out by Fischer in the synthesis of phenylalanine. Any aryl or alkyl radical may be substituted for the $C_6H_5CH_2$— radical of the benzyl chloride.

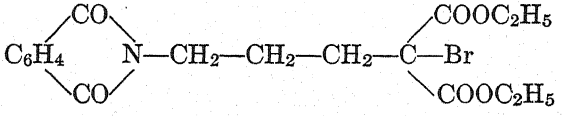
4. The synthesis from phthalimide. This synthesis was used by Fischer [26] in the preparation of ornithine. Potassium phthalimide,

[26] Fischer, E., Synthese der α-δ-di-aminovaleriansäure, *Ber.*, 34: 454–464 (1901).

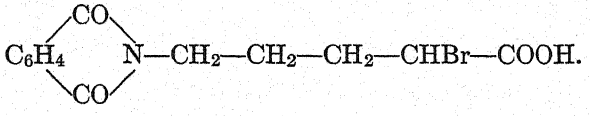
propylene bromide, and sodium malonic ester were combined to form γ-phthalimidopropyl malonic ester.



The γ-phthalimidopropyl malonic ester was then treated with bromine to form γ-phthalimidopropyl brom malonic ester:



This compound was then saponified to the substituted malonic acid and this by heating lost carbon dioxide, yielding δ-phthalimido-α-brom valerianic acid,



On treating this with ammonia, the bromine was replaced with (—NH_2) and hydrolysis with hydrochloric acid then split off phthalic acid and yielded inactive *ornithine*, α-δ-diaminovalerianic acid,

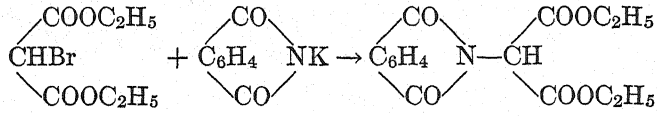
$$NH_2—CH_2—CH_2—CH_2—CH(NH_2)—COOH$$

The substituted brom malonic acid could not be directly converted into an amino compound, treatment with ammonia resulting in the formation of cyclic compounds. It was accordingly necessary to add the ammonia to the substituted brom valerianic acid.

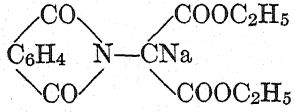
Sörensen [27] used phthalimide to add both the α and δ amino groups.

[27] Sörensen, S. P. L., Études sur la synthèse des acides amidés, *Compt. rend. trav. lab. Carlsberg*, 6: 1–60 (1903).

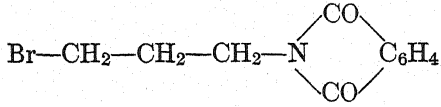
Brom malonic ester was combined with phthalimide potassium to form phthalimidomalonic ester:



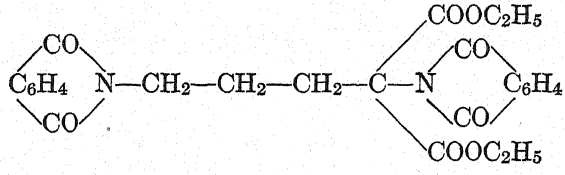
This compound was converted into phthalimidosodium malonic ester.



which was combined with γ-brom propylphthalimide.



yielding γ-phthalimidopropylphthalimidomalonic ester,

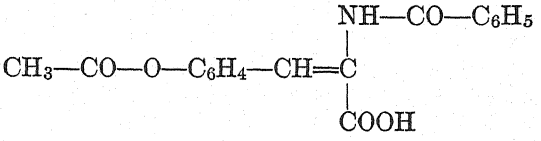


The ester groups were split off by saponification, the free acid lost carbon dioxide on heating, and the phthalic acid residues were removed by acid hydrolysis, forming the desired *ornithine* in good yield.

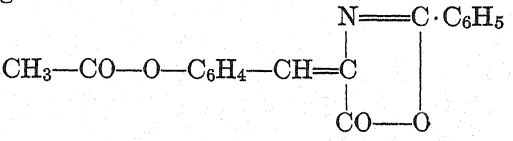
5. The hippuric acid synthesis. Erlenmeyer, in 1882, introduced the hippuric acid method, which has recently been improved by Lamb and Robson,[28] who used it to synthesize tyrosine and phenylalanine. It is the method by which Hoffmann-La Roche synthesize thyroxine. The reaction involves the condensation of the —CH_2— group of hippuric acid with the oxygen of an aldehyde in the presence of acetic anhydride. Thus starting with *p*-hydroxybenzaldehyde, hippuric

[28] Lamb, J., and Robson, W., The Erlenmeyer Synthesis of Amino-Acids, *Biochem. J.*, 25: 1231–1236 (1931).

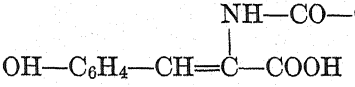
acid, and acetic anhydride, a compound is formed which has the structure:



This rearranges to the oxazolone:

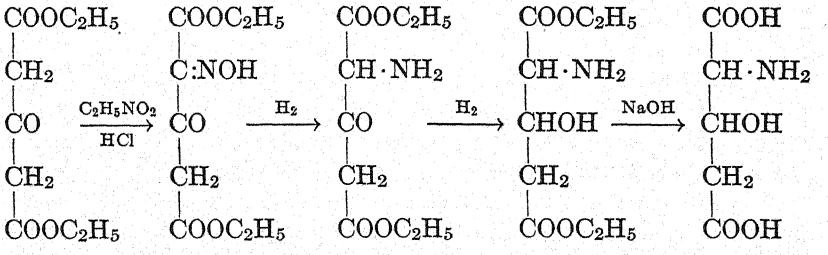


which on hydrolysis with sodium hydroxide yields a substituted benzoylaminoacrylic acid:



and this on reduction yields benzoyl-*d*-*l*-tyrosine from which the benzoyl group can be removed by acid hydrolysis. Yields by the hippuric acid method are fairly good, and it is a suitable method for the synthesis of phenylalanine, tyrosine, tryptophane, serine, etc.

6. The oximino synthesis. Isoleucine, leucine, aspartic, glutamic, and hydroxyglutamic acids have been synthesized by the reduction of the appropriate oximino compound. In the synthesis of hydroxyglutamic acid, Harington and Randall [29] treated the ester of acetone dicarboxylic acid (β-ketoglutaric acid) with ethyl nitrite to form the corresponding α-isonitroso derivative. This was then catalytically reduced in two stages, using a palladium-charcoal catalyst and in the second stage an added platinum catalyst. On saponification of the ester, *i*-hydroxyglutamic acid resulted.



[29] Harington, C. R., and Randall, S. S., Synthesis of *i*-β-Hydroxyglutamic Acid, with a Note on the Dissociation Constants by G. M. Richardson, *Biochem. J.*, 25: 1917–1930 (1931).

7. Other syntheses. In addition to those noted, a number of special syntheses have been used for the preparation of some particular amino acid. Those amino acids which contain the guanidine residue are usually prepared by the action of cyanamide upon the corresponding amine. Thus, cyanamide reacting on ornithine would produce arginine. Leuchs and Geiger's [30] synthesis of serine is another special reaction.

The methods used for the synthesis of the oxyamino acids are dependent very largely upon the particular amino acid which it is desired to synthesize. Only a single illustration will be given, *i.e.*, Leuchs and Geiger's synthesis of serine. The starting material for this synthesis is chloroacetal which is heated with sodium ethylate in an autoclave, yielding ethoxyacetal, this being hydrolyzed to ethoxyacetaldehyde, the amino acid being formed through the cyanhydrin synthesis, as indicated by the following reactions:

$$\mathrm{Cl{-}CH_2{-}CH{-}(O{-}C_2H_5)_2 + NaOEt = Et{-}O{-}CH_2{-}CH{-}(O{-}C_2H_5)_2 + NaCl}$$

$$\mathrm{Et{-}O{-}CH_2{-}CH{-}(O{-}C_2H_5)_2 + H_2SO_4 = Et{-}O{-}CH_2{-}CHO + 2C_2H_5OH}$$

$$\mathrm{Et{-}O{-}CH_2{-}CHO + HCN = Et{-}O{-}CH_2{-}CH(OH)CN}$$

$$\mathrm{Et{-}O{-}CH_2{-}CH(OH)CN + NH_3 = Et{-}O{-}CH_2{-}CH(NH_2)CN + H_2O}$$

$$\mathrm{Et{-}O{-}CH_2{-}CH(NH_2)CN \xrightarrow[\text{saponify}]{} Et{-}O{-}CH_2{-}CH(NH_2)COOH}$$

$$\mathrm{Et{-}O{-}CH_2{-}CH(NH_2)COOH \xrightarrow{\text{conc. HBr}} OH{-}CH_2{-}CH(NH_2)COOH}$$

(*i* serine)
(β-oxy-α-amino propionic acid)

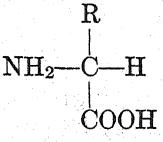
The above reactions are capable of producing a fair yield of inactive serine. In the author's laboratory, starting with 200 grams of chloroacetal, approximately 20 grams of pure inactive serine was obtained.

The Separation of the Racemic Mixture into Its Active Components.—With the exception of glycine (aminoacetic acid), all the naturally occurring amino acids contain an asymmetric carbon atom. As is usual in biological compounds, the naturally occurring amino acids are optically active, only the *dextro* or the *levo* form occurring in the protein. Miss Lloyd [31] comments on the rather striking fact that

[30] Leuchs, H., and Geiger, W., Über eine neue Synthese des Serins, *Ber.*, 39: 2644–2649 (1906).

[31] Lloyd, D. J., Colloidal Structure and Its Biological Significance, *Biol. Rev.*, 7: 254–273 (1932).

all the naturally occurring amino acids have the same spatial configuration with regard to the asymmetric carbon atom, i.e., the four groups R, H, COOH, and NH_2 always lie in the same relations to each other around the centrally placed tetrahedral carbon atom to which they are attached. It is difficult to depict these space relationships on a plane surface, but in the scheme usually employed the arrangement would be



The fact that all the naturally occurring amino acids have this same spatial configuration speaks strongly for a single primary mechanism as being involved in the synthesis of amino acids in nature.

Almost invariably, compounds which are synthesized in the organic laboratory, and which contain an asymmetric carbon atom, are what is known as a *racemic mixture,* where equal quantities of the *d*- and *l*-forms are present. Such a mixture is optically inactive, the levorotation of the *l*-form being neutralized by the dextrorotation of the *d*-form.

As we shall see later, there may be pronounced differences in solubility and in physiological action between the *d*- and *l*-forms of a chemical compound. Accordingly the task of a biological chemist is not completed when one of the syntheses noted above has yielded the pure amino acid. He must still separate the racemic mixture into its optically active components.

Three general methods are available for such separation.

1. *Mechanical Separation.*—Since the crystals of the *d*- and *l*-forms are mirror images of each other, it is possible to pick out the different crystals from a crystal mixture by hand, using forceps under a lens. Pasteur used this method to separate *d*- and *l*-tartaric acids. Unfortunately, amino acids rarely or never crystallize in large enough crystals to allow one to use this method of separation, so that the mechanical separation is of practically no use in protein research.

2. *The Biological Method.*—Biological organisms show a surprisingly high degree of specificity toward organic molecules. The yeasts, molds, and bacteria, as well as the higher animals, are usually capable of utilizing only one form of an optically active amino acid. In general, the optically active form which occurs in proteins is the one which is attacked by the biological organism.

In utilizing the biological method, the mixture of amino acids is inoculated with a pure culture of a bacterium, a fungus, or a yeast, in a culture medium, and the organism is allowed to grow and develop until one optically active isomer has been completely destroyed. The

solution is then worked up for the isolation of the optically active isomer which was not attacked by the biological organism. Unfortunately, while this method yields one of the optically active isomers, it is usually the optically active isomer which does not occur in nature. Accordingly, the biological method is rarely of great value in the isolation of the naturally occurring compound, that compound having been destroyed by the microorganism.

3. *The Chemical Method.*—The chemical method for separating racemic mixtures of amino acids depends upon the formation of a compound of the amino acid with some optically active substance. Alkaloids are very generally used for this purpose, inasmuch as they can be obtained in quantity and a high degree of purity at a comparatively low cost. Quinine, strychnine, brucine, and cinchonine are the alkaloids usually employed, inasmuch as they can be readily crystallized and purified. The alkaloids possess pronounced basic properties (*vide infra*), but the amino acids are not sufficiently acidic to combine directly with the alkaloids. It is accordingly necessary to intensify the acidic properties of the amino acid molecule. This is usually done by forming the benzoyl derivative of the amino acid.

$$R{-}CH(NH_2){-}COOH + C_6H_5COCl = R{-}CH(NHCOC_6H_5)COOH + HCl.$$

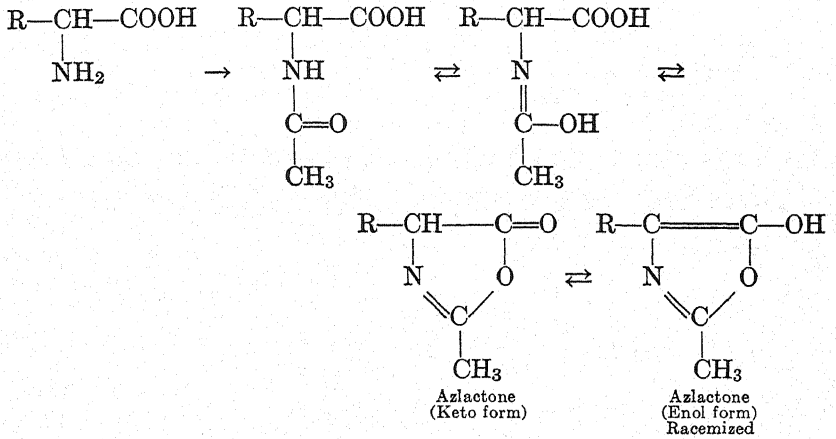
In this way the basicity of the amino group is masked, the acidity of the benzoyl derivative being intensified so that it forms a salt with the basic alkaloid.

The alkaloid which is selected and the benzoyl derivative are then mixed in the stoichiometrical proportions necessary for the formation of the alkaloid salt. Assuming that strychnine is the alkaloid chosen and that racemic phenylalanine is the amino acid which it is desired to separate into the optically active isomers, we would have a mixture of *l*-strychnine-*l*-benzoylphenylalanine and *l*-strychnine-*d*-benzoylphenylalanine. Such compounds will be found to differ in solubility. In some instances this difference is only slight; in others it may be very appreciable. The mixture is accordingly separated by a series of fractional crystallizations, retaining at one end of a series the most insoluble fraction and at the other end of the series the most soluble fraction. After a series of fractional crystallizations, testing the extreme fractions for the rotation of polarized light in a polarimeter, it will usually be found that one end of the series yields a constant dextrorotation, the other end of the series a constant levorotation, indicating that a complete separation has been obtained. When the separation is complete, the alkaloidal base is removed by appropriate means, the benzoyl derivative is hydrolyzed off by acid, and the optically active amino acid separated and recrystallized until it shows constant physical properties. If the crystals and the physical and chemical

properties of an amino acid so prepared are identical with the amino acid prepared from proteins, then and only then can one state with certainty that the naturally occurring amino acid has been synthesized.

A few of the naturally occurring amino acids, *e.g.*, cystine and the hydroxyamino acids, contain two asymmetric carbon atoms. Thus, in their synthesis four stereoisomers would be formed. The problem of the separation of these four isomers has not been solved, so far as the author is aware. Cystine itself, since the molecule is symmetrical, possesses the unique possibility of being able to exist in the *meso* form, and *l*-cystine, which had been racemized by boiling with hydrochloric acid, has been resolved [32] into *d*-cystine, *l*-cystine, and *meso*-cystine, in the latter compound one end of the molecule being derived from *d*-cysteine and the other from *l*-cysteine. *Meso*-cystine is strictly analogous in its optical behavior to *meso*-tartaric acid.

THE RACEMIZATION OF OPTICALLY ACTIVE AMINO ACIDS.—As we shall see later when we discuss the hydrolysis of proteins, hydrolysis with alkalies usually results in the racemization of part or all of the amino acids, and it is for this reason that alkaline hydrolysis is so seldom used. However, amino acids may be racemized by forming the acetyl derivative, the salts of which readily racemize.[33] The reactions which are involved are apparently:

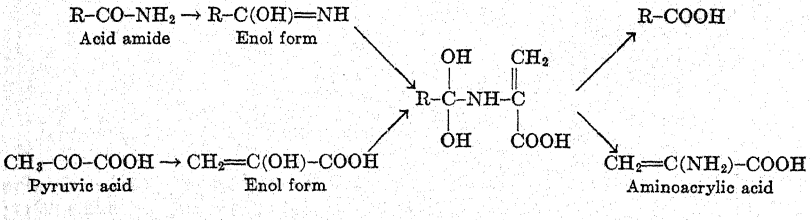


[32] Hollander, L., and du Vigneaud, V., The Resolution of Inactive Cystine and Isolation of Pure Dextrorotatory Cystine, *J. Biol. Chem.*, 94: 243–252 (1931); and Loring, H. S., and du Vigneaud, V., The Isolation of Two Isomeric Inactive Cystines, *Proc. Soc. Exptl. Biol. Med.*, 29: 41–42 (1931).

[33] du Vigneaud, V., and Sealock, R. R., The Racemization of Acetyl-*l*-Tryptophane, *J. Biol. Chem.*, 96: 511–517 (1932); du Vigneaud, V., and Meyer, C. E., The Racemization of Amino Acids in Aqueous Solution by Acetic Anhydride, *J. Biol. Chem.*, 98: 295–308 (1932); The Temporary Formation of the Azlactone Ring in the Racemization of Acyl Derivatives of Amino Acids with Acetic Anhydride, *J. Biol. Chem.*, 99: 143–151 (1932).

The essential characteristic of this reaction is that there must remain one hydrogen on the amino group after the acetyl derivative has been formed. Proline does not give the reaction. Neither does acetyl-N-methylphenylalanine, inasmuch as the enolization of the acetyl derivative cannot take place. This reaction appears to afford a very convenient method for the preparation of that form of the amino acid which does not occur in nature. It is possible that a somewhat similar mechanism is involved in the racemization of amino acids when proteins are hydrolyzed by alkali.

THE SYNTHESIS OF AMINO ACIDS IN NATURE.—The mechanism of the synthesis of amino acids in nature still remains to be proved. Animals are largely, if not entirely, dependent upon plant sources for the amino acids which they build into their body tissues. Although there is definite evidence that the animal organism can synthesize glycine and there is indirect evidence that the animal organism may be able to synthesize certain of the other amino acids, nevertheless there are at least ten amino acids which apparently the animal organism cannot synthesize and which it must obtain from its food sources, which in the last analysis are plant proteins. Numerous theories have been proposed for the mechanism involved in the synthesis of amino acids in the plant cell. All these have been critically reviewed by Björksten,[34] who concludes that the most probable mode of synthesis involves the interaction of the enol form of pyruvic acid with an acid amide. Theoretically ammonia could be substituted for the acid amide, but in a second paper [35] he rules out ammonia as a probable intermediate. Björksten suggests that the enol form of pyruvic acid condenses with the enol form of an acid amide and that the resulting product then breaks down to form an organic acid and aminoacrylic acid. The aminoacrylic acid is unstable and readily condenses with other compounds, which would account for the various amino acids which occur in nature.



The experiments which Björksten conducted lend weight to the above hypothesis, which seems to be the preferable one to accept, at least until more experimental data are available.

[34] Björksten, J., Zur Kenntnis der Synthese von Eiweissstoffen und ihrer Bausteine bei höheren Pflanzen, *Biochem. Z.*, 225: 1–80 (1930).

[35] Björksten, J., and Himberg, I., Spielt Ammoniak eine direkte Rolle bei der Eiweisssynthese höherer Pflanzen, *Biochem. Z.*, 225: 441–446 (1930).

CHAPTER XIII

POLYPEPTIDES

The name polypeptides was given by Fischer to compounds of two or more amino acids joined together by the peptide linkage. Polypeptides have been prepared by two general methods: (1) the partial hydrolysis of proteins, following some such procedure as allowing a protein to stand 12 to 48 hours in contact with *cold*, 60–80 per cent sulfuric acid, diluting the mixture with water, taking care that no appreciable rise in temperature occurs, neutralizing the sulfuric acid with barium hydroxide, again guarding against elevated temperature, filtering off the barium sulfate, and fractionally crystallizing the partial hydrolysis products in an attempt to isolate definite chemical compounds which could be later characterized so far as amino-acid content is concerned; and (2) the synthesis of polypeptides of known structure by the condensation of amino acids or amino-acid derivatives.

THE SYNTHESIS OF POLYPEPTIDES.—Schaal, in 1871, described an "anhydride" of aspartic acid, and Grimaux, in 1882, reported that this anhydride spontaneously transformed itself into "colloidal polyaspartic urea." Schiff, 1898–1899, on further investigation named the product polyaspartic acid. At about the same time, Schützenberger in a series of studies on "the synthesis of albuminoses and protein materials" treated various amino acids with phosphorus pentoxide and caused the formation of complex substances. Lilienfeld, in 1894, obtained complexes by treating amino-acid mixtures with various condensing agents, such as potassium pyrosulfate, formaldehyde, etc., and Balbiano and Frasciatti, in 1900, converted glycine into a horn-like substance by heating it with glycerol.

All the products obtained by the above workers were amorphous materials, having indifferent chemical characteristics. It was impossible to classify them or to determine their true relationship to the proteins.

Fischer early pointed out that, in order to arrive at a definite conclusion, some method must be devised whereby one could build up or tear down a chain of amino acids at will and that the reactions must be so controlled that the various steps of the process could be traced by the usual procedures of the organic laboratory.

Previous to Fischer, Curtius had busied himself with linking amino acids together, but in practically every instance he had used not the

free amino acid but the benzoyl derivative. In his studies, he obtained definite crystalline organic compounds. These, because they contained the benzoyl radical, were very different from the true polypeptides which were later to be synthesized.

As early as 1882, Curtius treated glycine silver with benzoyl chloride, obtaining, besides hippuric acid (benzoylaminoacetic acid), a substance which he called "hippurylaminoacetic acid" and which Fischer later showed to be the benzoyl derivative of the polypeptide glycylglycine. By the union of hippuric acid ester and glycine Curtius obtained a second compound, which he called "γ acid." This compound gave the biuret reactions, although the color was somewhat different from the biuret reaction as given by unaltered protein. Twenty-one years later, in the light afforded by Fischer's studies, Curtius repeated his experiments and found that the "γ acid" was in reality benzoylpentaglycylglycine. As early as 1884, Curtius had stated that besides hippuric acid a number of compounds could be obtained from glycine silver and benzoyl chloride, each compound differing from the other by one molecule of glycine with the elimination of one molecule of water. Accordingly Curtius must be credited with the first suggestion as to the peptide linkage and with the theoretical possibilities of linking amino acids together in chains to form polypeptides. Fischer pointed out that, though Curtius' statements were excellent theory, his experimental facts to prove the theory were lacking, except in the instances noted above.

Curtius observed another phenomenon, *i.e.*, that glycine ester in water solution could be transformed into glycine anhydride or diketopiperazine, the simplest member of the diketopiperazines,

$$2\ NH_2{-}CH_2{-}COOC_2H_5 \longrightarrow \begin{matrix} NH{-}CO{-}CH_2 \\ | \qquad\quad | \\ CH_2{-}CO{-}NH \end{matrix} + 2\ C_2H_5OH$$

(Diketopiperazine)

In 1901, Fischer and Fourneau [1] hydrolyzed glycine anhydride with acids and obtained the first polypeptide, *glycylglycine*. In a study of the various derivatives of glycylglycine, Fischer prepared the ethyl ester, and the carbethoxy ester of glycylglycine ester by the action of ethylchlorocarbonate:

$$C_2H_5{-}O{-}CO{-}Cl + NH_2{-}CH_2{-}CO{-}NH{-}CH_2{-}COOC_2H_5 =$$
$$C_2H_5{-}O{-}CO{-}NH{-}CH_2{-}CO{-}NH{-}CH_2{-}COOC_2H_5$$

(Carbethoxyglycylglycine ester)

A few months later he found that this carbethoxy ester would unite

[1] Fischer, E., and Fourneau, E., Über einige Derivate des Glykocolls, *Ber.*, 34: 2868–2877 (1901).

with the ester of another amino acid, *e.g.*, leucine ester, to give carbethoxy-glycylglycineleucine ester,

$$C_2H_5—O—CO—NH—CH_2—CO—NH—CH_2—CO—NH—CH(C_4H_9)CO—O—C_2H_5$$

the carbethoxy group of the glycylglycine ester intensifying the acidic properties of the compound, so that it unites directly with the leucine ester, with the elimination of a molecule of ethyl alcohol. At the same time Fischer announced a second polypeptide, leucylleucine, prepared by the partial hydrolysis of leucineimide, which itself had been described more than fifty years before.

Some months later, Curtius (1902) announced that by treating glycine with hippurylazide ($C_6H_5—CO—NH—CH_2—CO—N_3$) he could add one glycyl radical, and by repetition of the process he could lengthen the chain up to benzoylpentaglycylglycine. Thus, both Fischer and Curtius devised methods whereby chains of amino acids could be built up. In Fischer's method, however, the resulting compounds contained the carbethoxy group, and in Curtius' method, the benzoyl radical. Accordingly, the resulting compounds did not show physical properties similar to those possessed by the partial hydrolysis products of proteins.

In 1903, Fischer for the first time was able to prepare the *acid chloride* of glycine derivatives by treating carbethoxyglycine with thionyl chloride. This acid chloride would condense with an amino-acid ester to form a carbethoxydipeptide, which in turn could be converted into the acid chloride and further condensed with another molecule of an amino-acid ester. He was able to saponify the ester group of the peptides so formed, and obtained carbaminotriglycylglycine, having presumably the formula:

$$HOOC—NH—CH_2—CO—(NH—CH_2—CO)_2—NH—CH_2—COOH$$

This compound was nearer to a true tripeptide than were the benzoyl derivatives prepared by Curtius, or the carbethoxy derivatives prepared by Fischer, but still possessed a carboxyl group which could not be removed.

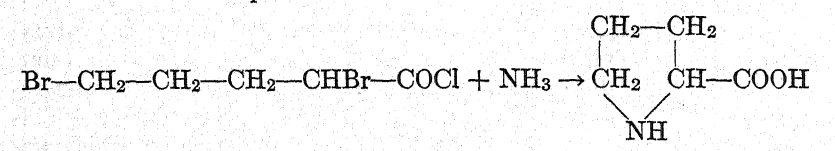
Accordingly, Fischer again altered his technic. Using chloracetyl chloride and glycylglycine ester, he obtained chloracetylglycylglycine, and on treating this compound with ammonia the chlorine was replaced with $—NH_2$, yielding the tripeptide, diglycylglycine. This compound he esterified and prepared the benzoyl derivative which was identical with a benzoyl product that Curtius had synthesized several years before.

In his study of the synthesis of amino acids, Fischer prepared a number of α-brom acids which could be easily converted into the corresponding acid chlorides by treatment with PCl_5. These acid chlorides he found would combine with amino acid esters to form derivatives

containing bromine, and when the bromine was removed by treatment with ammonia, polypeptides resulted. Thus, α-brom-isocaproyl chloride will combine with glycylglycine ester to form α-brom-isocaproylglycylglycine ester, which, on treatment with ammonia and saponification of the ester group, is converted into leucylglycylglycine. *Fischer further found that those derivatives of the polypeptides in which a halogen-containing acyl radical was attached to the amino group could be converted into the corresponding acid chloride by treatment with* PCl_5. Thus, α-brom-isocaproylglycylglycine could be converted by PCl_5 into α-brom-isocaproylglycylglycine chloride, and this in turn could be condensed with glycylglycine ester to form α-brom-isocaproyltriglycylglycine ester, and when this compound was treated with ammonia, the bromine was replaced with $—NH_2$, resulting in a pentapeptide, leucyltriglycylglycine; or prior to the treatment with ammonia, the compound could be again converted into an acid chloride and further condensations carried out. *It is impossible to prepare the acid chloride of an unsubstituted amino acid or of an unsubstituted polypeptide.* The acid chlorides can be obtained, however, provided that the amino group is masked with such radicals as carbethoxy, benzoyl, etc., or with radicals such as bromisocaproyl, brom-phenylacetyl, etc. It is obvious, of course, that, if one is to prepare derivatives corresponding to the natural amino acids of proteins, the halogen must be in the α position.

Fischer prepared a number of α-halogen acids for use in his polypeptide syntheses. Thus:

brom acetyl chloride treated with ammonia yields glycine;
α-brom propionyl chloride yields alanine;
α-brom butyryl chloride yields α-aminobutyric acid;
α-brom isocaproyl chloride yields leucine;
α-brom phenylacetyl chloride yields α-aminophenylacetic acid;
α-brom phenylhydrocinnamyl chloride yields phenylalanine;
α-δ-dibrom-valeryl chloride, instead of yielding an α-δ-diamino acid, condenses to form proline.



The use of α-halogen acids either to synthesize amino acids or as an aid in the synthesis of polypeptides was facilitated by the observation of Ward [2] that red phosphorus catalyzes the bromination of ali-

[2] Ward, C. F., The Bromination of Acids in the α-Position, *J. Chem. Soc.*, 121: 1161–1165 (1922).

phatic acids in the α-position. He suggests that this may be due to the following reactions:

$$R{-}CH_2{-}COOH + \text{red } P + Br_2 \rightarrow R{-}CH = C\begin{matrix}OH\\OH\end{matrix}$$

$$\rightarrow R{-}CHBr{-}C\begin{matrix}OH\\OH\\Br\end{matrix} \rightarrow R{-}CHBr{-}COOH + HBr.$$

The three methods which Fischer devised for the synthesis of polypeptides are:

1. The splitting of amino acid anhydrides or diketopiperazines by mild acid hydrolysis.
2. Condensing chloroacetyl chloride with amino-acid esters or esters of polypeptides and later treating the derivatives, so obtained, with ammonia in order to convert the chloroacetyl radical into a glycyl radical.
3. Forming acid chlorides of polypeptide derivatives which still contain in the molecule a halogenated acyl radical.

By use of this last process, Fischer [3] in 1907, prepared an octadecapeptide (18 amino acids), leucyltriglycylleucyltriglycylleucyloctaglycylglycine. He prepared this by combining brom-isocaproyldiglycylglycine chloride with pentaglycylglycine, yielding, when treated with ammonia, leucyloctaglycylglycine. This was further combined with another molecule of brom-isocaproyldiglycylglycine chloride, yielding, when treated with ammonia, leucyltriglycylleucyloctaglycylglycine. A further treatment with an additional molecule of brom-isocaproyldiglycylglycine chloride yielded, when the product was treated with ammonia, the octadecapeptide. This compound for many years had the highest molecular weight (1,213) of any compound prepared synthetically, the structure of which was accurately known. Later Abderhalden and Fodor [4] prepared a polypeptide containing 19 amino acids, *l*-leucyltriglycyl-*l*-leucyltriglycyl-*l*-leucyltriglycyl-*l*-leucylpentaglycylglycine, with a molecular weight of 1,326. If either one of the above polypeptides had contained amino acids other than glycine, such as tyrosine or phenylalanine, the molecular weight would have been about 3,000. Fischer describes his compound as a colorless, amor-

[3] Fischer, E., Synthese von Polypeptiden. XVII., *Ber.* 40: 1754–1767 (1907).
[4] Abderhalden, E., and Fodor, A., Synthese von hochmolekularen Polypeptiden aus Glykokoll und *l*-Leucin, *Ber.*, 49: 561–578 (1916).

phous powder, difficultly soluble in hot water to a solution which becomes turbid on standing; the solution foams, later is precipitated by saturation with ammonium sulfate, precipitated by tannins and by phosphotungstic acid, it gives the biuret test, but as would be expected, no other characteristic color tests. The turbidity in aqueous solution and its ease of foaming indicate that it is approaching colloidal dimensions, if indeed the solution is not a typical colloidal sol.

Bergmann and Zervas [5] introduced a fourth method for the synthesis of polypeptides which has proved to be very useful. The method consists essentially of combining the amino acid with carbobenzoxy chloride (C_6H_5—CH_2—O—CO—Cl) which readily unites with amino groups and protects them during the subsequent syntheses. The acid chloride of the carbobenzoxy derivative can then be formed using PCl_5, and this acid chloride is then condensed with the ester of another amino acid in the same way that Fischer condensed esters with his acid chlorides. The carbobenzoxy group can later be removed as toluene and carbon dioxide by catalytic hydrogenation at atmospheric pressure, leaving the free peptide. Bergmann has prepared a number of polypeptides by means of this reaction, and it is particularly adapted to the synthesis of polypeptides containing the dicarboxylic acids, such as *d*-glutamyl-*d*-glutamic acid and *l*-aspartyl-*l*-tyrosine. The method has also been used to synthesize the acid amides of both aspartic and glutamic acids and, in each instance, yielded either isoglutamine or isoasparagine, differing from the natural products in that the acid amide is formed on the α-carboxyl group.

THE LINKAGE IN POLYPEPTIDES.—The structure of the polypeptide molecule offers several possibilities of isomeric forms. Thus, there is the possibility of the polypeptide group undergoing keto⇌enol isomerism and of the terminal amino and carboxyl groups existing in the unionized state or ionized as zwitterions.

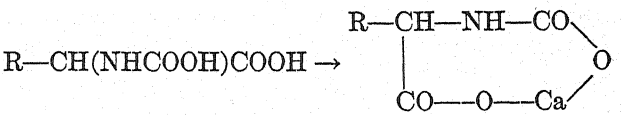
Certain polypeptides exist in more than one physical state. Thus, for example, leucyldiglycylglycine, when freshly prepared and in the amorphous state, is soluble in alcohol, but if the alcoholic solution is warmed, the compound separates in the form of crystals which are then essentially insoluble in alcohol. The chemical composition is unchanged, but by this procedure evidently there has been a shift from one form to another. The probable explanation of this change in solubility is that the unionized molecule has shifted into the zwitterion form, although the keto-enol shift may likewise be involved.

In order to show that various modifications of linkages exist, we have only to study the behavior of amino acids and polypeptides toward carbon dioxide in slightly alkaline aqueous solution. The so-

[5] Bergmann, M., and Zervas, L., Über ein allgemeines Verfahren der Peptid-Synthese, *Ber.*, 65B: 1192–1201 (1932).

dium, calcium, and barium salts of the monoamino acids react with carbon dioxide to form carbamino acids and carbamino salts.

$$R{-}CH(NH_2)COOH + CO_2 =$$



The monoamino acids have a ratio of nitrogen to added carbon dioxide of 1 : 1, tryptophane of 2 : 1, histidine of 3 : 1, and arginine of 4 : 1, indicating that only the free amino group reacts to form a carbamino acid and that the ring nitrogens or the nitrogen-containing groups of guanidine do not react to form carbamino acids. Siegfried and Liebermann [6] found that polypeptides also gave the carbamino reaction. Dipeptides, however, gave a ratio varying from 1.63 : 1 to 1.79 : 1, tripeptides a ratio of approximately 2.57 : 1, and tetrapeptides a ratio of 3.29 : 1 instead of the expected 2 : 1, 3 : 1, and 4 : 1 ratios, showing that some group other than the single, free amino group was functioning to some extent.

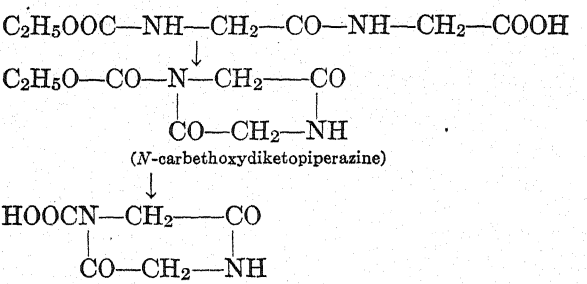
It should be noted at this point that this carbamino reaction is of importance in laboratory technic. Proteins, peptones, and amino acids all undergo the addition of carbon dioxide in alkaline solution, with the formation of carbamino acid derivatives. Accordingly, if one hydrolyzes the protein with sulfuric acid, adds an excess of barium hydroxide to remove the sulfuric acid, and then bubbles carbon dioxide through the solution to precipitate barium carbonate, it invariably happens that very appreciable amounts of barium are retained in solution, owing to the formation of soluble barium salts of the carbamino acids. When such a solution is boiled, a part of the carbamino acid breaks down, precipitating barium carbonate. This reaction, however, does not immediately go to completion. Accordingly, it is very difficult to free a solution, containing proteins, peptones, or amino acids, from barium by converting the barium into barium carbonate. Barium will often remain in the final concentrates and will have to be removed by adding an equivalent of sulfuric acid.

Leuchs and Manasse [7] in a study of this reaction noted that carbethoxyglycylglycine ester prepared from ethyl-chlor-carbonate and glycine ester, when hydrolyzed would not yield carbaminoglycylglycine

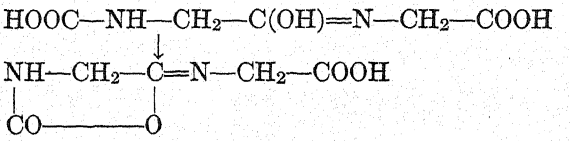
[6] Siegfried, M., and Liebermann, H., Über die Bindung von Kohlensäure durch amphotere Aminokörper, *Z. physiol. Chem.*, 54: 437–447 (1908).

[7] Leuchs, H., and Manasse, W., Über die Isomerie der Carbäthoxyl-glycyl-glycinester, *Ber.*, 40: 3235–3249 (1907). (*Cf.* also Leuchs, H., *Ber.*, 39: 857–861 (1906); Leuchs, H., and Geiger, W., *Ber.*, 41: 1721–1726 (1908); and Leuchs, H., and La Forge, F. B., *Ber.*, 41: 2586–2596 (1908).

ester as expected, but instead decomposed into glycine and carbon dioxide. They did, however, obtain from carbethoxyglycylglycine chloride an anhydride which formed a barium salt which yielded an anhydride of glycine, having different properties from the diketopiperazine prepared by Fischer, and which was isomeric with Fischer's diketopiperazine. On the hydrolysis of this anhydride, instead of obtaining the stable glycylglycinecarboxylic acid of Fischer, they obtained a very unstable compound. They represent the two isomeric acids and isomeric ring structures as follows; the *unstable* form of ring structure being derived from the acid ester:



which at once loses carbon dioxide and goes to diketopiperazine. The *stable* form of ring structure is derived from the acid:



a stable compound which does not lose carbon dioxide.

It will be noted that the stable ring compound is a *lactone*. Therefore, the stable form of glycylglycinecarbamino acid contains the grouping —N=COH— and is a *lactim* derivative, whereas the unstable form contains the grouping —NH—CO— and is a *lactam* derivative. The formulas of polypeptides are usually written in the lactam form. Inasmuch, however, as this form permits the addition of carbon dioxide and the formation of carbamino acids, whereas the lactim does not, it would appear that, in the light of the $N : CO_2$ ratios noted above, probably both the lactam and the lactim groupings occur in polypeptides. This is in agreement with the argument of Robertson [8] for a keto $\rightleftharpoons$ enol isomerism of the peptide linkage in proteins.

[8] Robertson, T. B., The Physical Chemistry of the Proteins, Longmans, Green and Company, New York (1918).

POLYPEPTIDES FROM PROTEINS.—As already noted, a number of workers have partially hydrolyzed proteins and have isolated polypeptides from the decomposition products. In a number of instances the amino acids present have been identified and their quantities accurately determined. Assuming that the product isolated is a tripeptide containing tyrosine, leucine, and alanine, there are six possible isomeric compounds having identical composition, *i.e.*:

1. Tyrosylleucylalanine.
2. Leucyltyrosylalanine.
3. Alanylleucyltyrosine.
4. Leucylalanyltyrosine.
5. Alanyltyrosylleucine.
6. Tyrosylalanylleucine.

Accordingly, the identification of a naturally occurring polypeptide or of a polypeptide isolated from protein decomposition products must usually be made by synthesizing the various possible isomers and by comparing the physical and chemical properties of the resulting synthetic products with the polypeptide which was isolated. In a number of instances this has been done, notably in the laboratories of Fischer and Abderhalden.

Sometimes, *e.g.*, in the determination of the structure of glutathione (glutamylcysteinylglycine), it is possible to determine the order of the amino acids in the molecule by chemical means.[9]

THE VALUE OF POLYPEPTIDE STUDIES.—The study of polypeptides has thrown much light upon protein structure.

1. Inasmuch as the synthetic polypeptides have in some instances been shown to be identical with the polypeptides isolated by the partial hydrolysis of proteins, we know definitely that the —NHCO— or —N=COH— group is present in the proteins. This fact, however, does not prove that the peptide linkage is the only linkage in proteins. In fact, polypeptide study indicates very strongly that it is not the only linkage, and though certain of the synthetic polypeptides are hydrolyzed by tryptic enzymes, none, so far as the author is aware, are hydrolyzed by the peptic enzymes. Fischer concluded that the chains of the polypeptides were not long enough for pepsin to act upon them, but it seems more probable that pepsin attacks some linkage other than the linkage in the peptide group. Trypsin hydrolyzes proteins to their constituent amino acids. Pepsin hydrolyzes proteins only to proteoses, peptones, and polypeptides.

[9] Kendall, E. C., McKenzie, B. F., and Mason, H. L., A Study of Glutathione. I. Its Preparation in Crystalline Form and Its Identification, *J. Biol. Chem.*, 84: 657–674 (1929); *cf.* also "The Configuration of Glutathione," *Proc. Staff Meetings, Mayo Clinic*, 4: 359–360 (Dec. 11, 1929).

2. The behavior of polypeptides toward tryptic and ereptic enzymes has shown in a striking manner the specificity of enzyme action. Trypsin hydrolyzes certain polypeptides; others it does not attack. In a racemic mixture of carbethoxyglycyl-*d*-*l*-leucine, it hydrolyzes the polypeptide of which *l*-leucine is a component but does not attack the polypeptide containing *d*-leucine. Alanylglycine is hydrolyzed by trypsin; glycylalanine is not. The position of the amino acid in the molecule has a marked influence on the ease of tryptic hydrolysis. When alanine is the acyl radical with glycine, alanine, or leucine, hydrolysis occurs, but when leucine, valine, or aminobutyric are the acyl radicals with alanine, no hydrolysis takes place. If the free carboxyl group is attached to tyrosine, cystine, or isoserine, they are readily split off by trypsin. In at least one instance examined by Fischer, where tyrosine was the acyl radical, no hydrolysis occurred.

The number of amino acids in the chain influences tryptic action. Triglycylglycine was not attacked by trypsin. Tetraglycylglycine was hydrolyzed. Triglycylglycine ester was likewise hydrolyzed.

Willstätter, Kuhn, and Waldschmidt-Leitz have been active in recent years in investigating the specific enzymes concerned in the hydrolysis of proteins, and erepsin has been shown to contain a mixture of enzymes. Among these are dipeptidases which attack only dipeptides, and in some instances specific dipeptides (note the dipeptidase of Bergmann, already discussed, which attacks dipeptides where the combination is with the imino group of proline). Another group is the tripeptidases, which again, in some instances, show marked specificity. A further group of the polypeptidases likewise contain enzymes having marked specificity toward synthetic or natural polypeptides. Long polypeptide chains may be differentially hydrolyzed at various linkages, depending upon the source of the enzyme employed. The net result of the enzyme studies appears to be a multiplicity of proteolytic enzymes, particularly in the ereptic group.

Natural proteins to some extent show similar differences toward enzymes. Fibrin, for example, is very easily digested by pepsin. Edestin is very slowly attacked, but is more easily attacked by trypsin than fibrin is.

Abderhalden studied the effect of other enzymes than those of the intestinal tract, on many polypeptides. Nearly all the enzymes which he studied were "tryptic type" but were not obtained from the pancreatic juice, instead representing the "Presssaft" of various organs. He notes that, *as a rule, the Presssaft of animal organs has a greater hydrolyzing action and less of a selective action than pancreatic trypsin.* He fed polypeptides to dogs and rabbits or injected them into the blood stream, and found that polypeptides which are not hydrolyzed by trypsin *in vitro* are burned in the animal body, the nitrogen being eliminated as urea. When, however, glycylglycine was injected subcu-

taneously into rabbits, it was eliminated in the urine as glycine, whereas, when glycine alone was injected, it was burned in the organism. Even the racemic compounds appear to be split and completely burned when fed, but how the hydrolysis and utilization come about is still uncertain.

3. Polypeptides have been of value in detecting the presence of proteolytic enzymes; those containing tyrosine, cystine, or tryptophane are particularly suited for this purpose. For example, glycyl-*l*-tyrosine is relatively soluble in water. The free tyrosine, however, is very insoluble. Similarly, the polypeptides containing cystine are usually relatively soluble. Cystine is almost insoluble. Polypeptides containing tryptophane do not give the characteristic rose-red color test of free tryptophane, when treated with dilute bromine water. When they are hydrolyzed, however, yielding free tryptophane, this very delicate color test can be used to indicate hydrolysis. Abderhalden has used rather extensively either the polypeptide, glycyl-*l*-tyrosine or "peptone roche" [10] for the identification of enzymatic action in tissues. If a section of an organ or tissue is covered with a solution of either glycyl-*l*-tyrosine or "peptone roche" and incubated at 37° for a time, tyrosine will crystallize out in stellate groups of needles upon those areas of the section where tryptic enzymes are present. Using this method, he found that proteolytic enzymes first made their appearance in chick embryos which were at the seven- or eight-day stage. Sections of 3.3-cm. pig embryos showed the presence of proteolytic enzymes in the liver and kidney areas. Sections of 3.2-cm. embryos, however, showed no evidences of the presence of proteolytic enzymes.

Polypeptides have been used to demonstrate the elaboration of proteolytic enzymes in the blood sera of animals injected with foreign protein. The normal blood sera of the horse or dog do not hydrolyze glycyl-*l*-tyrosine, although this polypeptide is hydrolyzed by the blood sera of the rabbit and the guinea pig. When, however, egg white or horse serum is injected into a dog and the dog allowed to become an anaphylactic reactor (*vide infra*), the blood serum acquires the property of hydrolyzing this polypeptide. Apparently the enzymes are elaborated in order to rid the blood stream of the foreign proteins, but the elaborated enzymes are not specific for the foreign protein. The elaboration of enzymes in the blood serum is not peculiar to the proteolytic enzymes. The intravenous injection of proteins will give rise to the formation of proteolytic enzymes. The intravenous injection of lactose or cane sugar will give rise to the elaboration of carbohydrases, and the injection of fat will give rise to the elaboration of lipases.

[10] Abderhalden, E., and Steinbeck, E., Weitere Untersuchungen über die Verwendbarkeit des Seidenpeptons zum Nachweis peptolytischer Fermente, *Z. physiol. Chem.*, 68: 312–316 (1910).

Polypeptides, however, do provide a convenient method of detecting the presence of relatively small quantities of proteolytic enzymes.

In special cases the enzymatic decomposition of polypeptides may be followed by placing the optically active polypeptide solution, together with the enzyme, in the tube of a polarimeter and noting changes in optical rotation. With this method, care must be taken to have comparison samples in which the enzyme alone and the polypeptide plus boiled enzyme are observed

CHAPTER XIV

THE ANALYSIS OF PROTEINS

Numerous methods have been proposed for the study of the amino-acid content of proteins and the identification of the various linkages or of the individual amino acids. By certain of these methods we can prove either the presence or the absence of individual amino acids or the presence or absence of certain groups of amino acids. Some of the methods give only qualitative information; others give quantitative.

It would be beyond the scope of the present work to consider any of these methods in sufficient detail to provide a complete manual for the laboratory worker. The discussion, therefore, will be limited to a consideration of the various technics which have been employed, with the literature citations so that the research worker may have the necessary information to obtain the exact technic and also some idea as to the limitations of the proposed method.

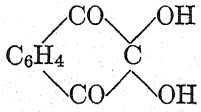
COLOR TESTS

Certain color reactions, characteristic of either amino acids or of linkages, have been proposed. These can be divided into two groups, those which are general for either linkages or for all amino acids, and those which are specific for some particular amino acid.

A. General. 1. *The Biuret Reaction.*—The biuret reaction is characteristic of the peptide linkage. When a solution of protein or of polypeptides containing this linkage is treated with a dilute solution of copper sulfate, after which a dilute solution of sodium hydroxide is added, a characteristic blue-violet to violet-pink color reaction occurs. This test is given by all native proteins and most of their split products. The longer chains of proteins, in general, yield a blue-violet coloration, but as the chain becomes shorter, the color grades more and more toward the pink. The color is due to the formation of a substituted biuret, RHN—CO—NH—CO—NHR′, which reacts with the copper hydroxide and alkali to form a colored complex. Urea, when heated, yields biuret, NH_2—CO—NH—CO—NH_2. Histidine gives the biuret reaction. Therefore, the test is not specific for the peptide linkage. However, both biuret and histidine give colors characteristic of the pink end of the color series, and as additional radicals are added to the biuret nucleus, the coloration becomes more and more a clear blue-violet. A blue-violet almost certainly indicates the presence

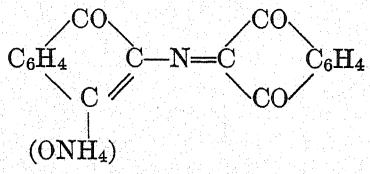
of protein or a long polypeptide chain. A single peptide linkage, such as that present in glycylglycine, will not give the biuret reaction. Three or more amino acids must be linked together in order to show the biuret test. The test is of value in determining the presence or absence of small quantities of protein in biological fluids.

2. *The Ninhydrine Reaction.*—When protein split products or α-amino acids or any compound containing an α-amino group are treated with "ninhydrine" (triketohydrindene hydrate),



in aqueous solution, color is developed, the color ranging from a clear, deep blue to a violet-pink or even red. This reaction was first noted by Ruhemann,[1] and has been studied by Harding and MacLean[2] as a quantitative method for the determination of amino acids. At least one free $—NH_2$ group must be present in order that color may be developed. Ninhydrine is probably the most delicate reagent for detecting the presence of protein or of an α-amino acid. With some amino acids, 1 part in 100,000 parts of water can be detected.

The reaction is much more delicate if carried out in the presence of a small amount of pyridine, probably owing to the buffer action of the pyridine. The reaction can be applied to the quantitative determination of an amino acid, provided that only a single amino acid is present in the solution. It is not, however, applicable to the quantitative estimation of amino acids when one is dealing with a mixture, since various amino acids give not only differences in the shade of color but differences in the depth of color per unit of the $—NH_2$ radical. Thus, using glycine as a standard, one can accurately determine quantitatively small amounts of glycine. The values for alanine, aspartic acid, etc., however, determined by a glycine standard, will be erroneous. The color change which is developed is that of a substituted ammonium salt of diketohydrindylidenediketohydrindamine:



[1] Ruhemann, S., Triketohydrindene Hydrate, *J. Chem. Soc.*, 97: 2025–2031 (1910).

[2] Harding, V. J., and MacLean, R. M., A Colorimetric Method for the Estimation of Amino-Acid α-Nitrogen, *J. Biol Chem.*, 20: 217–230 (1915).

Ninhydrine quantitatively decarboxylates α-amino acids when both the amino and carboxyl groups are free. Proline is also quantitatively decarboxylated. Aspartic acid loses both carboxyl groups. Glutamic acid loses one readily, the other only very slowly. Organic acids not containing an α-amino group do not lose CO_2. Van Slyke and Dillon [3] propose this as a quantitative measure of the carboxyl group in amino acids. They note that dipeptides do not react, and that urea and ammonia do not interfere. The CO_2 evolved is measured in Van Slyke's [4] manometric apparatus for organic carbon.

3. *Folin's Reagent.*—Folin [5] proposed β-naphthoquinone sulfonic acid as a general reagent for the colorimetric estimation of amino acids in blood. In the presence of strong alkalies and of an amino acid, this reagent develops a striking, deep red color. Ammonia likewise yields a color, but ammonia is readily removed, so that its interference would not be serious. Folin notes that urea, uric acid, creatinine, creatine, and hippuric acid do not yield colors. A color is developed with most of the nitrogen bases, such as the alkaloids or aniline, but as a rule these compounds are not present in solutions where one wishes to determine the presence or absence of amino acids.

B. Specific Reactions. 1. *The Millon Reaction.*—A red color is developed in many instances when a protein or a protein hydrolysate is heated with a solution of mercurous nitrate containing oxides of nitrogen or nitrous acid. *The red coloration is specific for the phenol group.* The reaction is, in general, considered as specific for tyrosine, although dopa and possibly the brom- or iodotyrosines would give the reaction. The test is capable of great delicacy when properly carried out, *i.e.*, when a considerable excess of the reagent is avoided.

2. *The Xanthoproteic Reaction.*—When protein is treated with nitric acid, a yellow coloration is produced which is intensified to orange on the addition of ammonium hydroxide. The yellow coloration so commonly produced on skin which has come in contact with nitric acid is an example of the xanthoproteic reaction and indicates the presence of *nitrated protein.* Nitration takes place on the benzene rings, and the reaction is, therefore, specific for *aromatic nuclei* which are easily nitrated (tryptophane or tyrosine). The benzene nucleus of phenylalanine is not readily nitrated. Accordingly, proteins containing no other aromatic nuclei excepting those in phenylalanine do not show the xanthoproteic reaction.

[3] Van Slyke, D. D., and Dillon, R. T., Gasometric Determination of Carboxyl Groups in Amino Acids, *Proc. Soc. Exptl. Biol. Med.*, 34: 362–363 (1936).

[4] Van Slyke, D. D., Page, I. H., and Kirk, E., A Manometric Micromethod for Determination of Carbon in Organic Compounds, *J. Biol. Chem.*, 102: 635–649 (1933).

[5] Folin, O., A System of Blood Analysis. Supplement III. A New Colorimetric Method for the Determination of the Amino-Acid Nitrogen in Blood, *J. Biol. Chem.*, 51: 377–391 (1922).

3. *Liebermann's Reaction.*—Most proteins, when heated in solution with concentrated hydrochloric acid, yield a violet or blue-black coloration. The production of such a color is specific for *tryptophane or indole derivatives* (*vide infra*).

4. *Acree-Rosenheim Reaction.*—If a protein or a mixture of amino acids is treated with a small amount of formaldehyde and then warmed with concentrated hydrochloric acid, a violet to black coloration is produced. This is another modification of the Liebermann reaction and is specific for tryptophane or indole derivatives. This reaction is used to detect the presence of formaldehyde added to milk. The casein of milk contains tryptophane. The formaldehyde accentuates the delicacy of the tryptophane reaction.

5. *The Adamkiewicz Reaction.*—This is a further modification of the reactions specific for tryptophane or indole derivatives. A small amount of acetic acid is added to a protein solution or to a mixture of amino acids, and concentrated sulfuric acid is carefully layered underneath the solution. A violet to black ring at the interface between the sulfuric acid and the solution is indicative of tryptophane. The acetic acid contains glyoxylic acid, COOH—CH—$(OH)_2$, which breaks down to form an aldehyde, the aldehyde being the actual reagent reacting with the tryptophane in the presence of acid, to form the color.

6. *The Benzaldehyde Reaction.*—Benzaldehyde, or preferably *p*-dimethylaminobenzaldehyde (Ehrlich's reagent), is specific for the indole nucleus and, therefore, for tryptophane. When a protein or a mixture of amino acids containing tryptophane is treated with benzaldehyde or Ehrlich's reagent in the presence of 20 per cent hydrochloric acid, an intense, clear blue coloration results. This again is merely another modification of the tryptophane-aldehyde reactions noted under Nos. 3, 4, and 5, above. The test is often employed to detect the presence of indole-forming bacteria.

7. *Reduced Sulfur Test.*—In many instances a black coloration is produced when a protein or a mixture of amino acids is heated with alkali and lead acetate. The coloration is due to the formation of black lead sulfide and appears to be specific for the presence of cystine or cysteine, or rather the groups —S—S— or —SH.

8. *The Sullivan Cystine Reaction.*—Sullivan[6] devised a very specific test for cystine or cysteine. It consists essentially of adding to the cystine hydrochloride 1–2 cc. of 1 per cent sodium cyanide solution made up in 0.8 *N* NaOH, then adding 1 cc. of a fresh 0.5 per cent aqueous solution of 1.2-naphthoquinone-4-sodium sulfonate, then adding 5 cc. of a 10–20 per cent Na_2SO_3 solution made up in 0.5 *N* NaOH, and allowing the mixture to stand at 20° C. for 30 minutes. Pure red

[6] Sullivan, M. X., Studies on the Biochemistry of Sulfur, *U. S. Pub. Health Repts.*, 44: 1421–1428, 1599–1608 (1929).

color is then developed by adding 1 cc. of a 2 per cent aqueous solution of sodium hyposulfite ($Na_2S_2O_4$). The color is specific for cystine or cysteine. A great variety of compounds were tested, but Sullivan found none that interfered. The —SH, —NH_2, and —COOH groups must all be free for the color to develop.

9. *The Molisch Test.*—The Molisch test is specific for carbohydrates. Many proteins contain a carbohydrate radical. In testing for such a radical, a trace of α-naphthol is added to a protein solution and this is layered over concentrated sulfuric acid. A pink to red coloration at the interface indicates the presence of carbohydrate. This test is probably dependent upon the formation of furfural from the carbohydrate.

10. *The Sakaguchi Test.*—Sakaguchi [7] proposed a color reaction which he claims to be specific for the *free-guanidine* group, and, therefore, for arginine. To 5 cc. of a 1 per cent solution of a protein, or of a 1 per cent solution of an amino-acid mixture containing arginine, is added 2 cc. of a 15 per cent solution of sodium hydroxide, followed by 5 cc. of a 0.15 per cent solution of α-naphthol, and the entire mixture treated with 0.3 *N* sodium hypochlorite. After standing at 2° to 4° C. for 40 minutes, the mixture is diluted and read against a standard solution in a colorimeter. The color which is developed is an intense red. Only compounds containing the free-guanidine group react. Accordingly the color test should be specific for arginine. In later papers Sakaguchi [8] shows that the guanidine group of arginine is free in most native proteins and also that the proteins differ widely in the rate with which arginine is set free when the proteins are hydrolyzed by enzymes or by acid or alkali.

11. *The Diacetyl Reaction.*—Harden and Norris [9] observed that, if a dilute solution of protein is mixed with a small amount of 10 per cent KOH solution and then one drop of a 1 per cent solution of diacetyl (CH_3—CO—CO—CH_3) is added, a pink color with a green fluorescence is developed. The reaction depends upon the presence of the arginine group in proteins, although the authors note that the exact shade of the color is somewhat dependent upon the compound in which the arginine group is substituted. In bacteriology this is known as the Voges-Proskauer test and is used for the testing for the presence of certain bacteria.[10]

[7] Sakaguchi, S., Über eine neue Farbenreaktion von Protein und Arginin, *J. Biochem.*, Tokyo, 5: 25–31 (1925); and Über die Bindungsweise und quantitative Bestimmung des Arginins im Proteinmoleküle, *ibid.*, 5: 133–142 (1925).

[8] Sakaguchi, S., Über Dearginoprotein, *J. Biochem.*, Tokyo, 5: 143–157 (1925); and Über die Spaltung des Proteinkörpers durch Alkali, *ibid.*, 5: 159–169 (1925).

[9] Harden, A., and Norris, D., The Diacetyl Reaction for Proteins, *J. Physiol.*, 42: 332–336 (1911).

[10] Werkman, C. H., An Improved Technic for the Voges-Proskauer Test, *J. Bact.*, 20: 121–125 (1930).

Hessler [11] studied the correlation between the intensity of the diacetyl reaction and the known arginine content of a series of fifteen proteins. He found that he could secure more accurate results by measuring the green color developed in acid than by measuring the pink color developed in alkalies. For fourteen of the fifteen proteins he found an almost exactly quantitative relationship between the diacetyl color and the known arginine content. For gliadin, for some unknown reason, he found color development with the diacetyl reagent to be inhibited.

THE QUANTITATIVE DETERMINATION OF AMINO AND CARBOXYL GROUPS IN AMINO ACIDS OR MIXTURES OF AMINO ACIDS

A. Van Slyke's Method.—Van Slyke [12] devised apparatus whereby one can determine quantitatively with a high degree of accuracy the primary amino nitrogen which is present in a protein, an amino acid, or a mixture of amino acids. The reaction involved is

$$R—NH_2 + HNO_2 = R—OH + N_2 + H_2O$$

where the nitrous acid is liberated by the action of acetic acid on sodium nitrite. The nitrogen gas evolved by the reaction is collected and measured. Van Slyke gives tables for the conversion of the volume of the nitrogen gas into milligrams of amino nitrogen. This method has proved of very great service not only in a study of the proteins but in the rapid analysis of amino acids and in the determination of relatively small amounts of amino acids in biological fluids. However, the ϵ-group of lysine reacts somewhat more slowly than the α-amino group. Only the primary amino groups react.

This method of Van Slyke for the determination of free amino nitrogen has been of particular value in a variety of problems. With the micro apparatus one can quantitatively determine 0.0005 gram or less of amino nitrogen. Thus, only extremely small quantities of an amino acid are necessary in order to determine the amino nitrogen content and accordingly its purity. The rate or extent of protein hydrolysis can be accurately followed by determining the free amino nitrogen at various intervals of time, since, when the peptide linkage is broken, a free amino group is formed. Similarly, the relative digestibility of proteins *in vitro*, *i.e.*, the rate of hydrolysis by a particular enzyme or by a mixture of enzymes, can be followed in a quantitative manner. The method affords information as to whether

[11] Hessler, L. E., The Diacetyl Reaction in Protein Study, M.S. thesis, University of Minnesota (1931).

[12] Van Slyke, D. D., A Method for Quantitative Determination of Aliphatic Amino Groups. Applications to the Study of Proteolysis and Proteolytic Products, *J. Biol. Chem.*, 9: 185–204 (1911); and The Quantitative Determination of Aliphatic Amino Groups, II. *ibid.*, 12: 275–284 (1912).

or not a given mixture contains proteolytic enzymes. If there is a progressive increase in free amino nitrogen, it can be taken as evidence that enzymatic action is present.

Van Slyke has shown that the ϵ $-NH_2$ group of lysine exists free in native proteins and that the free $-NH_2$ nitrogen content of native proteins can be correlated with their lysine content. There is still some doubt as to whether this is invariably the case. It appears, however, to afford at least a close approximation of the lysine content.

Plimmer,[13] in studying the Van Slyke reaction, investigated the behavior of a considerable variety of organic compounds. He points out that acid amides do not react with nitrous acid in the presence of acetic acid but react quantitatively in the presence of 2 *N* HCl. Similarly urea and guanidine react quantitatively in the presence of HCl but do not liberate nitrogen in the presence of acetic acid. *It is therefore essential that, when the Van Slyke amino nitrogen method is used, care be taken that no appreciable quantities of free mineral acids are present in the system.*

B. THE SÖRENSON TITRATION.—Sörensen[14] proposed a method whereby the carboxyl group of an amino acid could be titrated directly in aqueous solution with a standard alkali. Amino acids dissolve in water to form an essentially neutral solution, because of their zwitterion constitution. Sörensen added formaldehyde to the solution of amino acids, resulting in the formation of either a methylene derivative or an aldehyde ammonia.

$$R{-}CH(NH_3^+)COO^- + HCHO = R{-}CH(N{=}CH_2)COO^-H^+ + H_2O$$

or

$$R{-}CH(NH_3^+)COO^- + HCHO = R{-}CH(NH{-}CH_2OH)COO^-H^+$$

In this way the charge on the amino group is destroyed, and it is possible to titrate the carboxyl group with standard sodium hydroxide, using phenolphthalein as an indicator.

Sörensen's titration method is of value not only in quantitatively determining the carboxyl groups in an amino acid or a mixture of amino acids, but likewise in quantitatively determining amino acids in the presence of other organic acids. In this case, this mixture of amino acids and organic acids is titrated in aqueous solution with standard sodium hydroxide, using phenolphthalein as an indicator, until the organic acids which are present are completely neutralized. Neutral formaldehyde is then added and the solution further titrated with standard sodium hydroxide. Sörensen's method has proved of great value in enzyme studies, inasmuch as it permits one to follow

[13] Plimmer, R. H. A., The Action of Nitrous Acid upon Amides and Other "Amino"-compounds, *J. Chem. Soc.*, 127: 2651–2659 (1925).

[14] Sörensen, S. P. L., Enzymstudien, *Biochem. Z.*, 7: 45–101 (1907).

the rate at which the protein is being hydrolyzed, since the opening of the peptide linkage results in the formation of additional carboxyl groups.

C. FOREMAN'S TITRATION.—Foreman [15] noted that, when amino acids or even ammonium salts are titrated in 85 per cent ethyl alcohol, the ammonium radical or the amino groups do not react basic. The acid groups with which the ammonium radical is associated, or the carboxyl group of the amino acids, can accordingly be titrated directly in 85 per cent alcohol, using phenolphthalein as an indicator. A 0.1 *N* solution of ammonium chloride titrates in 85 per cent alcohol as though it were a 0.1 *N* solution of hydrochloric acid. It is sometimes more convenient to use Foreman's titration method than Sörensen's method. Martens [16] made a careful comparative study of the Van Slyke, Sörensen, and Foreman methods. He finds that, using phenolphthalein as an indicator, Foreman's method yields somewhat low results, Sörensen's method somewhat high results. He suggests a modification of Foreman's method, titrating the amino acid in 93 to 95 per cent alcohol and using thymolphthalein as an indicator. Under such conditions, the carboxyl groups can be determined with essentially the same degree of accuracy as is possible for the determination of the amino groups, using the Van Slyke method.

D. LINDERSTRØM-LANG'S TITRATION.—Linderstrøm-Lang [17] further modified the titration methods by titrating amino acids or proteins in 90–95 per cent acetone, using either standard alcoholic HCl or standard alcoholic NaOH, the HCl titrating the amino groups and the NaOH titrating the carboxyl groups. Apparently acetone like alcohol inhibits zwitterion formation.

E. WIDMARK AND LARSSON'S TITRATION.—Widmark and Larsson [18] suggest the estimation of amino acids by following the electrical conductivity of the amino-acid solution during the time that successive increments of sodium hydroxide are added to the solution. They used a dipping conductivity cell and plotted the conductivity values for the readings of the Wheatstone bridge, a/b, against the volume of standard sodium hydroxide which was added. There was a change in slope of the conductivity curve at the point where the carboxyl group of the amino acid was exactly neutralized. They present curves for the conductiometric titration of glycine, alanine, isoserine, α-amino-

[15] Foreman, F. W., Rapid Volumetric Methods for the Estimation of Amino-Acids, Organic Acids and Organic Bases, *Biochem. J.*, 14: 451–473 (1920).

[16] Martens, R., Considérations sur le dosage séparé des acides aminés et des polypetides dans les produits de digestion des protéines, *Bull. soc. chim. biol.*, 9: 454–482 (1927).

[17] Linderstrøm-Lang, K., Volumetric Determination of Amino Nitrogen, *Compt. rend. trav. lab. Carlsberg*, Vol. 17 (No. 4) (1927).

[18] Widmark, E. M., P., and Larsson, E. L., Bestimmung von Aminosäuren mittels konduktometrischer Titration, *Biochem. Z.*, 140: 284–294 (1923).

valerianic acid, leucine, norleucine, tryptophane, tyrosine, aspartic and glutamic acids, arginine, histidine hydrochloride, and lysine hydrochloride. The carboxyl group of arginine and histidine showed no evidence of combination with sodium hydroxide. The conductiometric titration of lysine did not yield a sharp end point. Figure 120 shows typical

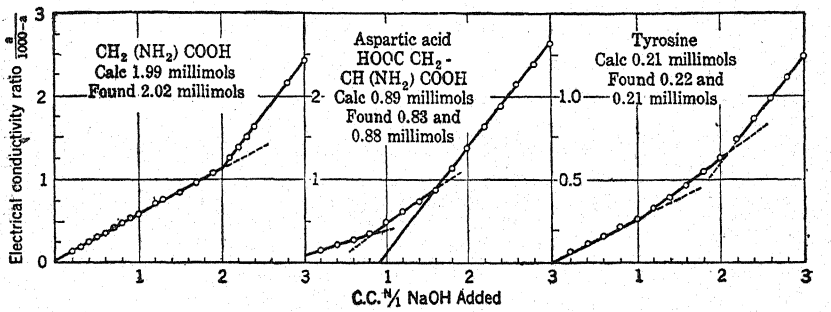


FIG. 120.—Conductiometric titration curves of amino acids with sodium hydroxide. (Data of Widmark and Larsson.)

conductiometric titration curves as obtained for glycine (one carboxyl group), aspartic acid (two carboxyl groups), and tyrosine (both the carboxyl and phenolic group reacting with the sodium hydroxide).

THE HYDROLYSIS OF PROTEINS

For the quantitative determination of individual amino acids, it is necessary to hydrolyze the protein into its constituent amino acids. Various methods have been proposed, *e.g.*, hydrolysis by acids, hydrolysis by alkalies, and hydrolysis by enzymes. Each has its advantages and disadvantages.

A. HYDROLYSIS BY ACIDS.—Constant-boiling (1.115 sp. g.) or approximately 20 per cent hydrochloric acid is most commonly used, although in some instances it is advantageous to use 25 per cent (by weight) sulfuric acid. Hydrolysis by 25 per cent sulfuric acid is usually somewhat slower than hydrolysis with constant-boiling hydrochloric acid but reaches the same completeness of hydrolysis.

Proteins differ widely in the rapidity with which hydrolysis reaches completion. In some instances, a protein may be completely hydrolyzed by boiling for 6 to 8 hours with 20 per cent hydrochloric acid. In other instances, as much as 24 hours' boiling may be required.

Various other acids have been tested. Hydrofluoric acid is unsuited for protein work, the dilute acid reacting too slowly, whereas the concentrated acid yields not simple amino acids but complex polypeptides or condensation products.

Formic acid causes only partial hydrolysis. Certain amino acids are hydrolyzed off, but apparently an equilibrium is reached fairly early in the process. In addition, there is the danger of forming formic derivatives with the amino group. The remaining organic acids are too weak to produce complete hydrolysis. Fodor and Epstein [19] studied the degradation of gelatin in the presence of acetic anhydride. In some instances, amino acids or amino-acid derivatives were found to be acetylated. A considerable fraction of the products isolated was in the form of polypeptides, indicating that even acetic anhydride will not completely hydrolyze proteins.

Nasset and Greenberg [20] measured the rate of the hydrolysis of casein in acid solution as evidenced by the formation of amino nitrogen and found it to be a second-order reaction obeying the equation

$$K = \frac{1}{t} \times \frac{x}{100 - x} \times \frac{1}{100} \tag{173}$$

where x = the percentage of protein hydrolyzed in time t.
They found hydrolysis to be proportional to the hydrogen-ion activity of the acid solutions.

B. Hydrolysis by Alkalies.—The alkalies, sodium hydroxide, potassium hydroxide, or barium hydroxide, cause very rapid and complete hydrolysis, although the strong alkalies bring about the decomposition, especially the deamination, of certain of the amino acids. Unfortunately, alkaline hydrolysis results in the racemization of the amino acids, so that they are isolated to a large extent in the optically inactive form. Because of this, alkaline hydrolysis is rarely used. It may, however, be used to advantage in the hydrolysis of proteins in which one wishes to determine tryptophane, inasmuch as tryptophane is destroyed by acid hydrolysis but is relatively stable toward alkaline hydrolysis.

C. Enzymatic Hydrolysis.—As noted earlier, pepsin hydrolyzes proteins only to proteoses, peptones, and polypeptides. Trypsin and erepsin, in general, hydrolyze proteins to the constituent amino acids, but the process is slow and, in many instances, not entirely complete. Accordingly, while tryptic hydrolysis is utilized in certain instances, it is not generally employed.

[19] Fodor, A., and Epstein, C., Über den Abbau der Gelatine durch Essigsäureanhydrid, *Z. physiol. Chem.*, 171: 222–241 (1927).

[20] Nasset, E. S., and Greenberg, D. M., The Rate of Hydrolysis of Casein in Acid Solutions as Measured by the Formation of Amino Nitrogen, *J. Am. Chem. Soc.*, 51: 836–841 (1929).

THE QUANTITATIVE STUDY OF THE HYDROLYTIC PRODUCTS OF PROTEINS

The methods for the quantitative study of the mixture obtained by the hydrolysis of proteins may be divided into two general groups, (A) those methods which are concerned with the identification of *groups of constituents*, and (B) those methods which are concerned with either the isolation or the quantitative estimation of *individual amino acids*.

A. GROUP ANALYSIS. 1. *Hausmann's Method.*—Hausmann [21] proposed the characterization of a protein by distributing the nitrogen content of its hydrolytic decomposition products into three groups, *i.e.*, ammonia nitrogen, basic nitrogen, and non-basic nitrogen.

Osborne [22] modified the Hausmann method by adding a fourth fraction, *i.e.*, that of humin nitrogen (older terminology = melanin nitrogen).

The Ammonia Nitrogen.—After acid hydrolysis, the excess of acid is removed by distillation, the solution containing the hydrolysis products is then rendered alkaline, using either magnesium oxide or preferably a suspension of calcium hydroxide, and the ammonia distilled into standard acid, preferably under a partial vacuum. The ammonia which is formed has been generally regarded as being derived from acid amide linkages, $—CONH_2$, and in many instances is referred to as the "amide nitrogen." However, a certain amount of deamination occurs under the usual conditions of acid hydrolysis and the true amide nitrogen value can be ascertained only by hydrolyzing for relatively short periods of time.[23] Under the usual conditions of hydrolysis as much as 20 per cent of the amide nitrogen of a 24-hour hydrolysate may be due to deamination. The deamination affects only the monoamino acids; the basic amino acids, arginine, histidine, and lysine, are not deaminized even by boiling for 6 weeks with constant-boiling hydrochloric acid.

The ammonia nitrogen has usually been ascribed to acid amide linkages attached to the free carboxyl group of one of the dicarboxylic acids. However, there appears to be no correlation [24] between the ammonia nitrogen of a protein hydrolysate and the dicarboxylic acids which can be isolated from that hydrolysate.

[21] Hausmann, W., Über die Vertheilung des Stickstoffs im Eiweissmolekül, *Z. physiol. Chem.*, 27: 95–108 (1899); II. *Ibid.*, 29: 136–145 (1900).

[22] Osborne, T. B., and Harris, I. F., Nitrogen in Protein Bodies, *J. Am. Chem. Soc.*, 25: 323–353 (1903).

[23] Gortner, R. A., and Holm, G. E., The Effect of Prolonged Acid Hydrolysis upon the Nitrogen Distribution of Fibrin with Especial Reference to the Ammonia Fraction, *J. Am. Chem. Soc.*, 39: 2736–2745 (1917).

[24] Gortner, R. A., Jr., The Relationship between the "Ammonia" Nitrogen and the Dicarboxylic Acids of Proteins, M.S. thesis, University of Minnesota (1934).

It is rather interesting to note that glutamine and asparagine differ widely in the ease with which ammonia is split off from the acid amide linkage. Thus, glutamine is completely hydrolyzed in 2 hours at 100° at *p*H 6.5, whereas asparagine is not affected under these conditions.[25] However, these relationships do not necessarily hold when the amino or carboxyl groups of the amino acid are combined in peptide or protein linkages.[26]

Since the ammonia nitrogen is split off from the protein in the early stages of protein hydrolysis, it should theoretically be possible to prepare highly "acid proteins" by splitting off the ammonia nitrogen and leaving the peptide linkages practically unaltered. No one, however, has succeeded in completely removing the ammonia nitrogen without partially breaking down some of the peptide linkages. Table LX shows

TABLE LX

PARTIAL HYDROLYSIS OF GLIADIN WITH DILUTE PHOSPHORIC ACID UNDER PRESSURE
(Analysis of Gliadin: Total N = 17.35%; free amino-N = unhydrolyzed 2.15% of Total N, completely hydrolyzed 58.6%; ammonia-N = 24.4% of total N)

(Unpublished Data—Gortner and Wiles)

Acid Concentration, Normality	Temperature, ° C.	Time, Minutes	Ammonia-N Liberated, % of Total Ammonia-N	Amino-N Liberated from Protein Linkages, % of Total Amino-N
0.005	170	180	76.3	5.32
0.010	170	60	57.9	2.51
0.010	170	120	77.1	7.05
0.010	170	180	92.1	13.84
0.030	183	40	96.3	5.60
0.040	180	40	95.5	9.73
0.050	180	40	106.2	9.14
0.10	170	60	102.0	7.12

the data resulting from such an attempt, using gliadin as the protein and hydrolyzing with dilute phosphoric acid under pressure.

The Humin Nitrogen.—The humin (melanin) of a protein hydrolysate is the black, amorphous material which separates when proteins are hydrolyzed with strong acids. Humin is formed by the condensa-

[25] Vickery, H. B., Pucher, G. W., Clark, H. E., Chibnall, A. C., and Westall, R. G., The Determination of Glutamine in the Presence of Asparagine, *Biochem. J.*, 29: 2710–2720 (1935).

[26] Chibnall, A. C., and Westall, R. G., The Estimation of Glutamine in the Presence of Asparagine, *Biochem. J.*, 26: 122–132 (1932).

tion [27] of tryptophane with an aldehyde. The reaction apparently is with the indole nucleus, the condensation presumably taking place on the α-hydrogen. The colors produced in the Liebermann, Acree-Rosenheim, and Adamkiewicz reactions are due to the early stages of humin formation. The evidence presented by Gortner and his co-workers shows clearly that proteins contain either an aldehyde group or some other group which reacts as an aldehyde in condensing with tryptophane, resulting in the formation of humin. The nature of the compound to which this reactive group is attached, however, remains still to be determined. It may be that some compound similar to djenkolic acid, which breaks down yielding formaldehyde as one of the products, is present in most proteins.

The humin nitrogen determination can be so conducted as to be a quantitative measure of the tryptophane content of a protein. Thus, when fibrin was hydrolyzed in the presence of increasing amounts of formaldehyde, the black, acid-insoluble humin nitrogen was increased from 9.60 mg. per 3 grams of fibrin, where no formaldehyde was added, to a maximum of 19.02 mg. of acid-insoluble humin nitrogen, where 0.10 gram of formaldehyde in the form of trioxymethylene was added. The addition of larger amounts of trioxymethylene sharply decreased the amount of acid-insoluble humin nitrogen. The 19.02 mg. of acid-insoluble humin nitrogen appears to be a practically quantitative measure of the tryptophane nitrogen which was present in the original 3 grams of fibrin.

The humin nitrogen, as obtained in an ordinary protein hydrolysate, bears little or no relationship to the true tryptophane nitrogen content of the protein, inasmuch as the humin formation depends upon two variables, the tryptophane content and the presence of an adequate amount of aldehyde. Accordingly, the humin formed in a protein hydrolysis as ordinarily carried out, though dependent upon the tryptophane content of the protein, is not a quantitative measure of that trytophane content.

Humin formation apparently involves the interaction of the indole nucleus with three molecules of aldehyde. The first molecule of aldehyde appears to condense to form a substituted indolidene-methane. The second molecule of aldehyde forms a compound of the rosindole type. These compounds are highly colored, and certain compounds of this type have been utilized as dyestuffs. The third molecule of aldehyde condenses with the rosindole type of compound with the elimina-

[27] Gortner, R. A., *et al*, The Origin of the Humin Formed by the Acid Hydrolysis of Proteins, I–VIII, *J. Am. Chem. Soc.*, 37: 1630–1636 (1915); *J. Biol. Chem.*, 26: 177–204 (1916); *J. Am. Chem. Soc.*, 39: 2477–2501 (1917); *ibid.*, 42: 632–640, 821–827, 2378–2385 (1920); *ibid.*, 45: 550–553 (1923); *ibid.*, 46: 1224–1246 (1924).

tion of a molecule of water and forms the "humin." The type of structure involved still remains to be elucidated. Apparently the α-amino-propionic acid side chain of tryptophane is not involved in the reaction, since at maximum humin formation in the presence of aldehyde one-half of the total nitrogen of the humin is still free α-amino nitrogen.

The Basic Nitrogen.—In the Hausmann method the basic nitrogen is determined by precipitating the diamino acids, arginine, histidine, lysine, and cystine, with phosphotungstic acid in the presence of an excess of hydrochloric acid. The phosphotungstates of these amino acids are nearly insoluble. The phosphotungstate precipitate is filtered off, washed with a dilute solution of phosphotungstic acid in dilute hydrochloric acid, and the nitrogen in the entire precipitate determined by the Kjeldahl method.

The Non-Basic Nitrogen.—The non-basic nitrogen is determined by kjeldahling an aliquot of the filtrate from the phosphotungstate precipitate. This fraction of nitrogen represents the monoamino-monocarboxylic acids and the monoaminodicarboxylic acids.

Thus, the Hausmann method divides the nitrogen of a protein hydrolysate into four fractions. The *advantages* of the method are that it is rapid, that it requires only a small amount of protein, 0.5 gram to 1 gram, and that by this method one can obtain rather definite information as to the ratio existing between the diamino acids and the monoamino acids comprising the protein molecule. The *disadvantages* of the method are that the method gives no clue as to the presence or absence of any individual amino acid with the possible exception of tryptophane, and that there are many proteins having somewhat similar content of basic and non-basic amino acids, such proteins being more or less indistinguishable from each other by the Hausmann technic.

2. *Van Slyke's Method.*—Van Slyke [28] made use of his method for determining amino nitrogen and the ratio between total nitrogen and free-amino nitrogen in certain of the amino acids, together with the Hausmann procedure, in order to estimate quantitatively certain of the amino acids in proteins. In Van Slyke's method the protein is hydrolyzed, and the *acid amide nitrogen* and *humin nitrogen* determined essentially as in Hausmann's method.

The *diamino acids* are precipitated with phosphotungstic acid in 5 per cent (by weight) hydrochloric acid, and the basic phosphotungstates are filtered off and washed with dilute phosphotungstic acid in

[28] Van Slyke, D. D., The Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino-Acids, *J. Biol. Chem.*, 10: 15–55 (1911); Improvements in the Method for Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino-Acids, *ibid.*, 22: 281–285 (1915); and Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino-Acids. Correction, *ibid.*, 23: 411 (1915).

dilute hydrochloric acid. This precipitate is then dissolved in very dilute alkali and decomposed by the addition of 20 per cent barium chloride solution, the insoluble barium phosphotungstate is filtered off and washed, and the filtrate containing the basic amino acids is slightly acidified and concentrated to a definite volume. This filtrate contains the arginine, histidine, lysine, and a part of the cystine which were present in the original protein. These four amino acids are then estimated as follows:

a. Total sulfur is determined on an aliquot of this solution as barium sulfate by the method of Benedict,[29] as modified by Denis,[30] and from the weight of barium sulfate obtained, the *cystine nitrogen* content is calculated.

b. The total nitrogen present in the basic fraction is determined on an aliquot of the solution of the bases.

c. The free-amino nitrogen content is determined by Van Slyke's method in an aliquot of the solution.

d. Sufficient potassium hydroxide is added to an aliquot of the bases to make a solution containing 50 per cent of potassium hydroxide. This solution is boiled for 6 hours under such conditions that the volume remains constant and any ammonia which is evolved is collected in standard acid. By this process arginine is decomposed into urea and ornithine, the urea being further broken up into ammonia and carbon dioxide.

Consequently half of the arginine nitrogen has been evolved as ammonia. From the amount of nitrogen which is evolved as ammonia, the quantity of *arginine nitrogen* in the original solution is calculated.

e. We now have values for cystine, arginine, total nitrogen, and amino nitrogen. The amount of *histidine* which is present is secured by a mathematical calculation, inasmuch as histidine and arginine are the only basic amino acids which contain non-amino nitrogen. Two-thirds of the histidine nitrogen and three-fourths of the arginine nitrogen are non-amino nitrogen (the $—NH_2$ group of the guanidine nucleus does not react with nitrous acid). Accordingly the non-amino nitrogen of the bases minus three-fourths of the arginine nitrogen is equal to two-thirds of the histidine nitrogen, which permits us to calculate the amount of *histidine nitrogen* in the basic fraction.

f. The *lysine nitrogen* is finally obtained by subtracting from the total nitrogen, the sum of the arginine nitrogen plus the histidine nitrogen plus the cystine nitrogen.

Van Slyke further differentiates the nitrogen in the filtrate from the bases by use of his amino nitrogen apparatus into (1) amino nitro-

[29] Benedict, S. R., The Estimation of Total Sulfur in Urine, *J. Biol. Chem.*, 6: 363–371 (1909).

[30] Denis, W., The Determination of Total Sulphur in Urine, *J. Biol. Chem.*, 8: 401–403 (1910).

gen, and (2) non-amino nitrogen, the former being derived from the monoaminomonocarboxylic and monoaminodicarboxylic acids, the latter being derived from proline, oxyproline, and in part from tryptophane.

Although the essential details of the Van Slyke original method have remained unchanged, certain modifications in technic have been introduced [31] which make either for ease in manipulation or for greater accuracy in the analytical results. More recently a micro method [32] has been proposed which yields the same fractions as the original Van Slyke method, but where as little as 25 mg. of nitrogen are necessary.

Van Slyke's method has been used very extensively in protein studies. Larmour [33] collected the various analyses which had been published up to 1928 and subjected them to statistical analyses. We are not at this point concerned with his statistical findings. His paper, however, can conveniently be used as a source of reference for an extensive series of protein analyses.

Van Slyke's method has the *advantages* of requiring a relatively small amount of protein, 3 grams or less, and of permitting the more or less quantitative determination of arginine, histidine, and lysine. The cystine value is too low, inasmuch as cystine is partly decomposed when boiled with acids, the portion which is not decomposed being either racemized or converted into an isomeric form, the phosphotungstate of which is appreciably soluble.[34]

In a critical study [35] of Van Slyke's method, using mixtures of known amino acids, the conclusion reached was that the histidine and lysine values may be somewhat in error when tryptophane and proline are present. The arginine which was added was determined within experimental error. The cystine nitrogen in three experiments was 64.5, 62.1, and 73.3 per cent of the cystine nitrogen added. The histidine values were satisfactory in the absence of tryptophane or proline but were high in the presence of either one or both of these amino

[31] Plimmer, R. H. A., and Rosedale, J. L., Analysis of Proteins. V. Van Slyke's Method of Determination of Nitrogen Distribution. VII. Direct Estimation of Arginine, *Biochem. J.*, 19: 1004–1014; 1020–1021 (1925); *cf.* also Cavett, J. W., A Modification of the Van Slyke Nitrogen Distribution Method, *J. Biol. Chem.*, 95: 335–343 (1932).

[32] Rosedale, J. L., and Da Silva, Gladys Anne, The Amino-Acids of Tissues. VI. Determination of the Basic Amino-Acids in Small Quantities of Protein, *Biochem. J.*, 26: 369–376 (1932).

[33] Larmour, R. K., Correlations Between the Total Nitrogen of the Bases and Arginine Nitrogen and between Total Nitrogen of the Bases and Lysine Nitrogen, of Various Proteins, *Trans. Roy. Soc. Can.*, Section V, 349–363 (1928).

[34] Hoffman, W. F., and Gortner, R. A., Sulfur in Proteins. I. The Effect of Acid Hydrolysis upon Cystine, *J. Am. Chem. Soc.*, 44: 341–360 (1922).

[35] Gortner, R. A., and Sandstrom, W. M., Proline and Tryptophane as Factors Influencing the Accuracy of Van Slyke's Method for the Determination of Nitrogen Distribution in Proteins, *J. Am. Chem. Soc.*, 47: 1663–1671 (1925).

acids. The lysine values were only slightly high and might well have been attributed to experimental errors.

The *disadvantages* of Van Slyke's method lie in the fact that it gives us no insight into the composition of the group of monoamino-monocarboxylic and monoaminodicarboxylic acids. In a number of instances workers have used the Van Slyke method on various biological materials which were not pure proteins, and, though valuable *comparative results* may be obtained by such technic, it should be pointed out that the "arginine nitrogen," "histidine nitrogen," "lysine nitrogen," etc., so obtained do not necessarily bear any relationship to the true arginine, histidine, lysine, etc., content of the material, because any compound which breaks down under the action of strong alkali to yield ammonia would be calculated as arginine nitrogen, the balance of the non-amino nitrogen would be calculated as histidine nitrogen, etc., and purines, pyrimidines, etc., would distribute themselves, if present, among the arginine, histidine, and lysine fractions.

B. Isolation and Identification of Individual Amino Acids. 1. *Fischer's Ester Method.*—In Fischer's study of amino-acid derivatives he observed that the ethyl esters of the monoaminomonocarboxylic and monoaminodicarboxylic acids could be distilled *in vacuo* without appreciable decomposition. It is upon this observation that he based his ester method.

The protein is hydrolyzed by hydrochloric acid; the excess of hydrochloric acid is removed by distillation; the mixture of amino acid hydrochlorides is concentrated; and the concentrated solution is saturated with gaseous hydrochloric acid. On standing at a low temperature, *glutamic acid hydrochloride* crystallizes out. This is filtered off and recrystallized from concentrated hydrochloric acid. The free glutamic acid can be obtained from this hydrochloride (any other amino acid chloride where the amino acid has a low basic dissociation constant acts similarly) by suspending the hydrochloride in alcohol and adding pyridine or aniline,[36] so as to combine with the hydrochloric acid. The free amino acid will then crystallize from the alcoholic solution.

The remaining amino acids are then converted into their ethyl esters by boiling with absolute ethyl alcohol in the presence of hydrochloric acid or zinc chloride. On concentrating and cooling the mixture of esters, *glycine ester hydrochloride* crystallizes out and can be removed by filtration. The excess of hydrochloric acid in the remaining mixture of esters is then neutralized by some appropriate technic. The preferable method is to determine accurately the amount of hydrochloric acid which is present and add an exact equivalent of sodium

[36] Benedict, H. C., A Note on the Use of Aniline in the Preparation of Amino Acids, *J. Am. Chem. Soc.*, 51: 2277 (1929); *cf.* also Cox, G. J., The Preparation of *d*-Arginine Monohydrochloride, *J. Biol. Chem.*, 78: 475–479 (1928).

ethylate dissolved in absolute alcohol. The free esters are now soluble in absolute ether. The ethereal solution is dried and submitted to vacuum distillation, the following fractions being secured,

60° (10 mm.) = glycine, alanine, leucine, proline.
60°–100° (10 mm.) = valine, leucine, proline.
100°–130° (0.5 mm.) = leucine and proline.
130°–180° (0.5 mm.) = phenylalanine, glutamic acid, aspartic acid, and serine.

It will be noted that there is a considerable overlapping of amino acids in the various fractions. The residue which does not distil contains arginine, histidine, lysine, tyrosine, cystine, oxyproline, and various anhydrides, such as leucinimide, and other diketopiperazines formed by secondary reactions.

The ester fractions, as obtained in the vacuum distillation, are hydrolyzed by boiling with water, and the individual amino acids separated by a process of fractional crystallization.

It is obvious that such a method is far from quantitative. Osborne and Jones,[37] after many years of experience in working with Fischer's ester method, undertook a study of the method, in order to ascertain the errors involved. They list the following sources of error: (1) incomplete hydrolysis, (2) loss of amino acids, due to the formation of humin, (3) incomplete esterification, (4) the decomposition of the esters by hydrolysis prior to distillation, (5) unavoidable loss in separating the amino acids by fractional crystallization. To the above, we must undoubtedly add (6) the conversion of amino acid esters into diketopiperazine derivatives. In this experiment, they mixed pure amino acids (omitting the boiling with hydrochloric acid for 24 hours), esterified the mixture, liberated the free esters, and distilled *in vacuo*. The various fractions were then worked up for their amino-acid content. Table LXI shows that the distillable esters were only 73 per cent, and the total recovery was only 66.17 per cent, of the theory. This probably explains why most of the analyses of proteins by Fischer's ester method range from 40 to 60 per cent of the weight of the protein taken.

Actually the weight of amino acids theoretically obtainable exceeds considerably the weight of the protein from which the amino acids are derived, inasmuch as the elements of water are added to each peptide linkage. A protein should yield from 110 to 120 per cent of its weight of amino acids. The isolation of 60 per cent of the amino acids in a protein analysis means, therefore, that approximately only half of the amino acids which are actually present have been accounted for.

[37] Osborne, T. B., and Jones, D. Breese, A Consideration of the Sources of Loss in Analyzing the Products of Protein Hydrolysis, *Am. J. Physiol.*, 26: 305–328 (1910).

TABLE LXI

RECOVERY OF AMINO ACIDS FROM A KNOWN MIXTURE BY FISCHER'S ESTER METHOD
(DATA OF OSBORNE AND JONES)

Amino Acids Taken		Amino Acids Recovered	
	Grams	Grams	Per Cent
Alanine	16.00	7.34	45.88
Valine	4.00	1.64	41.00
Leucine	85.00	69.36	81.60
Proline	31.00	22.56	72.77
Phenylalanine	26.00	18.07	69.51
Aspartic acid	6.00	2.55	42.50
Glutaminic acid	120.20	83.26	69.39
Tyrosine	16.00	7.97	49.81
Arginine	5.50	3.57	64.91
Histidine	2.04	0.80	39.21
Serine	2.00	0.00	0.00
Ammonia	14.31		
Total	328.05	217.12	66.17

2. *Dakin's Method.*—Dakin,[38] in 1918, introduced a new method which has advantages either in replacing or in supplementing Fischer's method. In Dakin's method, the protein is hydrolyzed with sulfuric acid; the sulfuric acid is quantitatively removed with barium hydroxide; and the aqueous solution of amino acids concentrated until crystallization of amino acids begins. This thick, semi-crystalline mass is then extracted in a continuous liquid extractor with *n*-butyl alcohol. The amino acids themselves, with the exception of proline, are insoluble in anhydrous *n*-butyl alcohol, but the monoaminomonocarboxylic acids are slightly soluble in *n*-butyl alcohol saturated with water. The aqueous butyl alcohol distils at a lower temperature than does absolute butyl alcohol. Accordingly there is left in the receiving flask a crystalline mass of the monoaminomonocarboxylic acids. The absolute butyl alcohol containing the proline is removed from this crystalline mass, and the crystalline mass is subjected to fractional crystallization for its individual constituents, or it may be esterified and the esters distilled as in Fischer's method.

The solution which has been extracted with butyl alcohol contains the diamino acids, the dicarboxylic acids, tyrosine, and diketopiperazines. The basic amino acids can be removed by precipitation with phosphotungstic acid. Tyrosine, because of its insolubility in water,

[38] Dakin, H. D., On Amino-Acids, *Biochem. J.*, 12: 290–317 (1918); and Amino-Acids of Gelatin, *J. Biol. Chem.*, 44: 499–529 (1920).

can be readily obtained, and the dicarboxylic acids are then separated by fractional crystallization.

It was by use of this modification that Dakin found in excess of 10 per cent of a new amino acid, β-hydroxyglutamic acid, in casein.

It is rather interesting to note that, although the dicarboxylic acids cannot be extracted by butyl alcohol from a neutral aqueous solution, they are extracted by butyl alcohol from an aqueous solution having a *p*H of approximately 3.0.

Dakin's method of extraction with butyl alcohol affords in many instances a valuable procedure for the purification of an individual amino acid. Amino acids which cannot be readily obtained in a crystalline form from aqueous solution readily crystallize when extracted from the aqueous solution with normal butyl alcohol in a continuous extractor.

3. *The Brazier-Schryver Method.*—Miss Brazier,[39] working in Schryver's laboratory on hydrolytic products of zein, introduced a radically new method for the separation of the amino acids. The protein is hydrolyzed with sulfuric acid, the acid is removed with baryta, and the ammonia is aspirated off. Any residual barium is then quantitatively removed, and the amino acids are converted into their copper salts by boiling with copper carbonate. The mixture is then completely dehydrated in the presence of an excess of copper carbonate and then shaken in a mechanical shaker with water containing additional copper carbonate. The copper salts of leucine, phenylalanine, and aspartic acid remain as an insoluble residue.

The insoluble residue is treated with H_2S to remove the copper, rendered alkaline with barium hydroxide, and the addition of three volumes of 95 per cent alcohol precipitates the aspartic acid as barium aspartate. The barium is removed from the filtrate of the barium aspartate with sulfuric acid, and leucine crystallizes from the concentrated solution. The filtrate from the leucine is converted into the zinc salts with freshly precipitated $Zn(OH)_2$. Zinc leucine is only slightly soluble in cold water; zinc phenylalanine is readily soluble. The zinc salts are decomposed with H_2S to obtain the free amino acids.

The copper salts which were soluble in water are evaporated to dryness and dehydrated with acetone. The residue is then extracted with absolute methyl alcohol. The copper salts of alanine, glutamic acid, tyrosine, arginine, histidine, and glycine are insoluble in absolute methyl alcohol. The copper salts of valine, hydroxyvaline, proline, and a dipeptide, prolylphenylalanine, were soluble in absolute methyl alcohol.

Glutamic acid was separated from the insoluble residue as barium glutamate. Tyrosine was crystallized from the filtrate of the barium glutamate. Histidine was removed from this filtrate as the insoluble

[39] Brazier, Mary A. B., A New Method for the Separation of the Products of Protein Hydrolysis, *Biochem. J.*, 24: 1188–1198 (1930).

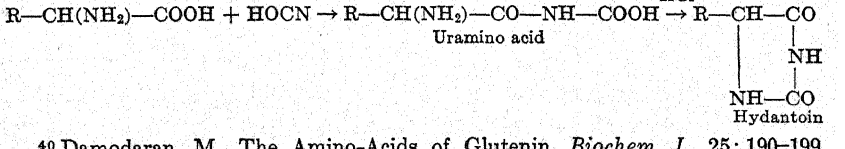
$Zn(OH)_2$—$HgCl_2$ complex. Arginine was separated as the flavianate, and the remaining alanine extracted from the residue by Dakin's butyl alcohol method.

Those copper salts which were soluble in methyl alcohol were converted into the free acids with H_2S. Prolylphenylalanine crystallized from the aqueous solution. Proline was separated as the picrate or as the double compound with cadmium chloride. By reconversion through the copper and zinc salts, zinc valine was separated as insoluble in absolute ethyl alcohol, and zinc hydroxyvaline as soluble.

In Miss Brazier's analysis of zein, she accounted for 93.5 per cent of all the nitrogen in the original protein, the balance being lost in inorganic precipitates which were discarded. She recovered 87.89 per cent of the nitrogen as ammonia or as pure amino acid fractions. In almost every instance, the amount of amino acid which is reported is higher than the highest value reported in the previous literature.

In a subsequent paper from Schryver's laboratory, Damodaran [40] applied the copper-zinc salt method to the analysis of glutenin. Here again, in almost every instance, the yield of the isolated amino acids exceeded the highest yield reported in earlier literature. However, when one reads Damodaran's paper, one finds that the actual technic for the isolation of a particular amino acid has been varied somewhat from the specifications given by Miss Brazier, and in the author's laboratory it has been found that this is apparently a characteristic of the method, *i.e.*, for any given mixture of amino acids, a given set of conditions will produce a standard set of fractions, but if a new protein is used which contains amino acids not present in the previous protein, fractions having properties different from those anticipated may result. Apparently, unless more specific conditions are laid down, or at least until a wide variety of proteins have been analyzed by this method, the analysis of each new protein through the series of metallic salts must be regarded almost as a research problem. Jukes [41] has suggested the removal of the dicarboxylic acids and the basic amino acids prior to the fractionation of the remaining amino acids as the copper and zinc salts, and believes that this simplifies the procedure.

4. *Boyd's Uramino and Hydantoin Method.*—When amino acids are treated with potassium cyanate, they readily form uramino acids, and these, when treated with dilute hydrochloric acid, are easily transformed into hydantoins.



[40] Damodaran, M., The Amino-Acids of Glutenin, *Biochem. J.*, 25: 190–199 (1931).

[41] Jukes, T. H., The Fractionation of the Amino Acids of Livetin, *J. Biol. Chem.*, 103: 425–437 (1933).

The uramino acids and hydantoins, in contrast to the amino acids, are relatively insoluble in water and in general readily soluble in organic solvents. Boyd [42] made a study of the solubilities of the various uramino and hydantoin derivatives and proposes a method for the fractionation of the amino acids from proteins based on his solubility studies.

The proteins were hydrolyzed by boiling with hydrochloric acid, the excess of acid was removed by concentrating *in vacuo*, followed by a neutralization of the hydrolysate, and the amino acids were converted into the uramino acids by boiling with potassium cyanate. The cold solution was then carefully acidified to the turning point of Congo red, and the uramino acids derived from leucine, isoleucine, and phenylalanine separated as a precipitate. These were then converted into the hydantoins and separated by differential solubility.

The uramino acids remaining in solution were separated into two fractions based on their solubility or insolubility in alcohol at *p*H 4. The uramino acids from the monoaminomonocarboxylic acids are soluble in alcohol at *p*H 4, whereas the remaining amino acids are relatively insoluble. From those which are soluble in the alcohol, the hydantoins of leucine, isoleucine, valine, phenylalanine, alanine, proline, and hydroxyproline can be removed by their solubilities in ether or chloroform. The hydantoin of tyrosine is insoluble in ether and remains behind in practically pure crystalline form. The details of the separation by differential solubilities of the various hydantoins is too involved to be readily abstracted, but it appears as though Boyd's method may be a valuable adjunct to other methods in problems involving protein analysis.

The advantages of the method lie in the fact that the melting points and crystallographic properties of the uramino and hydantoin derivatives are exceedingly characteristic, so that the amino acid from which they are derived is excellently characterized by the physical properties of these derivatives. The amino acid can be regenerated by the hydrolysis of the uramino or hydantoin derivatives, the disadvantages of this process of securing the amino acids being that the regenerated amino acid is usually racemized.

5. *Kossel's Separation of the Bases.*—Kossel and Kutscher [43] suggested a method whereby the diamino acids arginine, histidine, and lysine could be prepared in pure form and quantitatively determined with a fairly high degree of precision.

[42] Boyd, W. J., The Isolation of Amino-Acids in the Form of the Corresponding Carbamido-Acids and Hydantoins. I. The Derivatives of the Mono-Amino-Monocarboxylic Acids, *Biochem. J.*, 27: 1838–1848 (1933).

[43] Kossel, A., and Kutscher, F., Beiträge zur Kenntniss der Eiweisskörper, *Z. physiol. Chem.*, 31:165–214 (1900).

The protein is hydrolyzed with sulfuric acid, and the sulfuric acid later removed quantitatively with barium hydroxide. A hot, saturated solution of silver sulfate is then added to the aqueous solution of amino acids until a drop of the silver amino-acid solution turns to a brown color on the addition of barium hydroxide, indicating the formation of silver oxide and accordingly an excess of silver in the solution. The solution is then saturated with powdered barium hydroxide, precipitating the silver salts of *arginine* and *histidine*. *Lysine* remains in solution and can be precipitated later by phosphotungstic acid.

The precipitate containing the silver salts of arginine and histidine is filtered off, the silver removed with hydrogen sulfide, and the solution of amino acids filtered from the resulting precipitate of silver sulfide. The excess of hydrogen sulfide is removed by aeration. Hot, concentrated silver sulfate solution is again added until an excess of silver is present. At this point barium hydroxide solution is added until the solution is neutral. Histidine silver precipitates at neutrality, leaving the arginine silver in solution. The histidine silver is filtered off, decomposed with hydrogen sulfide, and the solution of the free amino acid concentrated to a small volume. The addition of a hot, saturated alcoholic solution of picrolonic acid precipitates the histidine practically quantitatively as the histidine picrolonate.

The filtrate from the histidine, containing the arginine silver, is saturated with powdered barium hydroxide, precipitating arginine silver. This precipitate is filtered off, the silver removed with hydrogen sulfide, the excess of barium quantitatively removed with sulfuric acid, and the aqueous solution of arginine concentrated to a small volume and the picrolonate prepared as in the case of histidine.

The solution containing the lysine is freed from silver with hydrogen sulfide, acidified with sulfuric acid, and the lysine precipitated as lysine phosphotungstate. This precipitate is decomposed with baryta, the excess of barium is quantitatively removed with either carbon dioxide or an exact equivalent of sulfuric acid, the solution containing the free lysine is evaporated to a small volume, and the lysine separated as lysine picrate.

This method has been modified in some particulars by later investigators. Vickery and Leavenworth[44] controlled the separation of arginine and histidine silver by hydrogen-ion-concentration measurements. Histidine silver is completely precipitated at a *p*H of 7.0, whereas arginine silver remains completely in solution. They recommend double precipitation, thus insuring the absence of traces of the

[44] Vickery, H. B., and Leavenworth, C. S., Modifications of the Method for the Determination of the Basic Amino Acids of Proteins. The Bases of Edestin, *J. Biol. Chem.*, 76: 707–722 (1928); *cf.* also *J. Biol. Chem.*, 79: 377–388 (1928); *ibid.*, 93: 105–112 (1931); and *Biochem. J.*, 26: 1101–1106 (1932).

other amino acids, and recommend the separation of arginine as arginine flavianate[45] (2.4-dinitro-1-naphthol-7-sulfonate).

As already noted, Kossel's method is essentially quantitative, although the results are somewhat lower than the analyses indicated by Van Slyke's method. Its great *advantage* lies in the fact that by this method the basic amino acids can be rather readily prepared.

6. *The Carbamate Method.*—Buston and Schryver,[46] in 1921, announced a new method for the separation of amino acids from the products of protein hydrolysis. The method depends upon the formation of the carbamino derivatives which we have already discussed. The solution containing the amino acids is treated with an excess of barium hydroxide and saturated with carbon dioxide, forming the barium carbamates. Later Kingston and Schryver[47] proposed a general scheme for the separation of the hydrolytic products of proteins, based on the carbamate reaction. The dicarboxylic acids can be quantitatively precipitated as the barium carbamates, when three volumes of alcohol are added to the amino-acid solution which has been treated with barium hydroxide and carbon dioxide. When the amino-acid solutions are sufficiently concentrated, practically all the amino acids can be precipitated in the presence of alcohol, as the barium carbamates, with the exception of proline which remains in the alcoholic mother liquor.

The carbamate method offers particular advantages for the separation of the dicarboxylic acids from the monocarboxylic acids, and it appears probable that its chief usefulness will be in connection with a study of the dicarboxylic fraction.

7. *The Separation of the Basic Amino Acids and the Dicarboxylic Amino Acids by Electrodialysis.*—Foster and Schmidt[48] utilized electrodialysis to separate the diamino and the dicarboxylic amino acids from the monoaminomonocarboxylic acids. The protein is hydrolyzed with sulfuric acid, the sulfuric acid quantitatively removed with baryta, and the aqueous solution of the amino acids is placed in the center compartment of an electrodialyzer separated from the anode and the cathode chambers by collodion or parchment membranes.

[45] Pratt, A. E., The Preparation of *d*-Arginine Carbonate, *J. Biol. Chem.*, 67: 351–356 (1926).

[46] Buston, H. W., and Schryver, S. B., A Method for the Separation of Amino-Acids from the Products of Hydrolysis of Proteins and Other Sources, *Biochem. J.*, 15: 636–642 (1921).

[47] Kingston, H. L., and Schryver, S. B., Investigations on Gelatin. Pt. III. The Separation of the Products of Hydrolysis of Gelatin by the Carbamate Method, *Biochem. J.*, 18: 1070–1078 (1924).

[48] Foster, G. L., and Schmidt, C. L. A., The Separation of the Hexone Bases from Certain Protein Hydrolysates by Electrolysis, *J. Biol. Chem.*, 56: 545–553 (1923); The Separation of the Dicarboxylic Amino Acids from Certain Protein Hydrolysates by Electrical Transport, *J. Am. Chem. Soc.*, 48: 1709–1714 (1926).

Using carbon electrodes and adjusting the acidity of the protein hydrolysate to a *p*H of 5.5, arginine, histidine, and lysine migrated almost quantitatively to the cathode compartment. At a *p*H of 5.7 only arginine and lysine migrated, the histidine remaining in the center compartment. By repeating the electrolysis on the cathode liquor, the basic amino acids were separated almost completely from the other amino acids of the protein hydrolysate. The dicarboxylic acids and proline migrated to the anode, and when the solution in the anode compartment was again electrodialyzed, only small quantities of monoaminomonocarboxylic acids were found in the anode compartment.

A more recent study [49] gives explicit details for the preparation of the hexone bases using electrical transport. The method of electrodialysis can be used to advantage in supplementing certain of the methods which we have already discussed.

8. *The Preparation and Determination of Cystine.*—Cystine can be rather readily prepared from proteins, such as hair or wool, which contain a high percentage of cystine. The method [50, 51] usually employed is that of hydrolyzing the protein with acid, concentrating the mixture of amino acids, and neutralizing the free hydrochloric acid, preferably completing the neutralization with sodium acetate which acts as a buffer, thus avoiding the presence of free alkali which very rapidly decomposes cystine. The hydrogen-ion concentration at this point is sufficiently reduced so that cystine spontaneously crystallizes, owing to its very low solubility.

Cystine is very readily altered by alkalies, with the exception of ammonia. Even washing the hair with a hot 1 per cent solution of sodium carbonate will prevent the isolation [52] of any appreciable amount of cystine from such material. It is rather interesting that the effect of alkali upon cystine is apparently not a function of the hydroxyl-ion concentration, inasmuch as concentrated alkalies produce less decomposition than dilute alkaline solutions.[53] The greatest decomposition comes about when cystine is boiled with just enough alkali to form the disodium or dipotassium salt. Barium hydroxide in equivalent concentration causes much greater decomposition than sodium hydroxide. When sodium plumbate is present in the alkaline solu-

[49] Cox, G. J., King, Harriette, and Berg, C. P., The Preparation of Lysine, Histidine, and Arginine from Hydrolyzed Blood Corpuscle Paste by Electrical Transport, *J. Biol. Chem.*, 81: 755–764 (1929).

[50] Folin, O., On the Preparation of Cystin, *J. Biol. Chem.*, 8: 9–10 (1910).

[51] Gortner, R. A., and Hoffman, W. F., *l*-Cystine, Organic Syntheses, Vol. V, pp. 39–41, John Wiley & Sons, New York (1925).

[52] Hoffman, W. F., Sulfur in Proteins. II. The Effect of Mild Alkaline Hydrolysis upon Hair, *J. Biol. Chem.*, 65: 251–254 (1925).

[53] Thor, C. J. B., and Gortner, R. A., Sulfur in Proteins. V. The Effect of Alkalies upon Cystine, with Special Reference to the Action of Sodium Hydroxide, *J. Biol. Chem.*, 99: 383–403 (1933).

tion, decomposition goes practically to completion. Cystine can be boiled for 12 hours in 4 N NaOH without undergoing more than 50 per cent decomposition, and practically all this decomposition occurs within the first hour of boiling. Apparently some decomposition product stabilizes the residual cystine, so that an equilibrium is reached and long-continued boiling only slightly displaces this equilibrium. The decomposition with alkali involves the simultaneous removal of both nitrogen and sulfur from the cystine molecule.

When cystine is boiled with strong mineral acids, it is racemized,[54] giving rise to both *i*-cystine and *meso*-cystine. In addition there is some slight (± 10 per cent) decomposition. The phosphotungstates of the racemic cystine have a greater solubility than the phosphotungstate of *l*-cystine, which probably accounts for the low cystine values in Van Slyke's nitrogen distribution method.

Okuda[55] proposes the estimation of cystine by oxidation with standard potassium bromate solution. A solution of cystine in 10 per cent hydrochloric acid is treated with 0.1 N potassium bromate in the presence of potassium bromide, the cystine being oxidized to cysteic acid according to the following reactions:

$$KBrO_3 + 5\ KBr + 6\ HCl = 6\ KCl + 3\ H_2O + 3\ Br_2$$

$$\underset{\text{Cystine}}{C_6H_{12}O_4N_2S_2} + 10\ Br + 6\ H_2O = 2\ HOOC{-}\underset{\underset{\underset{\text{Cysteic acid}}{NH_2}}{|}}{CH}{-}CH_2{-}SO_2{-}OH + 10\ HBr$$

Okuda notes that, of all the amino acids precipitated by phosphotungstic acid, histidine is the only one that interferes in the above titration and that the rate of reaction of histidine with bromine is much slower than that of cystine, so that histidine does not interfere to any great extent.

In most protein studies cystine is determined colorimetrically either by Sullivan's reaction (*vide supra*) or by the phosphotungstic-phosphomolybdic reagent and technic of Folin and Marenzi.[56]

9. *The Preparation and Determination of Tyrosine.*—Owing to its relative insolubility in water (1 : 2450 at 17°), tyrosine can be rather readily isolated by direct crystallization. The protein is hydrolyzed

[54] Hoffman, W. F., and Gortner, R. A., Sulfur in Proteins. I. The Effect of Acid Hydrolysis Upon Cystine, *J. Am. Chem. Soc.*, 44: 341–360 (1922).

[55] Okuda, Y., A Method for the Determination of Cystine, *J. Coll. Agr., Imp. Univ. Tokyo*, 7: 69–76 (1919).

[56] Folin, O., and Marenzi, A. D., Tyrosine and Tryptophane Determinations in One-Tenth Gram of Protein, *J. Biol. Chem.*, 83: 89–102 (1929); An Improved Colorimetric Method for the Determination of Cystine in Proteins, *J. Biol. Chem.*, 83: 103–108 (1929); The Preparation of Uric Acid Reagent Completely Free from Phenol Reagent, *J. Biol. Chem.*, 83: 109–113 (1929).

with sulfuric acid, the sulfuric acid quantitatively removed with baryta, and the precipitated barium sulfate thoroughly washed with hot water. The filtrates are concentrated to a small volume, and the tyrosine allowed to crystallize. The crude crystals are recrystallized from hot water.

Various colorimetric methods have been proposed for the determination of tyrosine. The phenol reagent of Folin and Denis consisting of phosphotungstic-phosphomolybdic acid, as adapted by Folin and Looney,[57] is suitable for the estimation of tyrosine when proper precautions are taken.

10. *The Preparation and Determination of Tryptophane.*—The actual isolation of tryptophane in the crystalline form is a rather laborious procedure. Since tryptophane is destroyed by acid hydrolysis, the amino acid must be liberated by digestion with trypsin, after which it is precipitated as the mercury salt in a dilute sulfuric acid solution and the mercury salt worked up for the crystalline amino acid. Although tryptophane is rather readily isolated from casein by this procedure, its isolation from other proteins is rarely accomplished, possibly because the tryptic digest contains polypeptides or other decomposition products which interfere with the procedure.

Tryptophane is usually estimated colorimetrically, using either the reagent of Folin and Marenzi (*loc. cit.*) or the *p*-dimethylaminobenzaldehyde reagent as adapted by May and Rose.[58]

11. *The Prepararation and Determination of Glycine.*—We have already indicated that glycine ester hydrochloride can be rather readily obtained in the Fischer ester method. The isolation, however, is not quantitative when only small amounts are present in the protein. Patton[59] uses the color developed in the interaction of glycine and *o*-phthalic dialdehyde for the estimation and finds that the color is specific for glycine.

Bergmann and Fox[60] propose the use of potassium trioxalatochromiate, $[Cr(C_2O_4)_3]K_3 \cdot 3H_2O$, which they found to be specific for the precipitation of glycine and which can be used either for the isolation of glycine or for its quantitative estimation.

12. *The Preparation and Determination of Proline.*—The double salt of ammonium thiocyanate and potassium dichromate, known as

[57] Folin, O., and Looney, J. M., Colorimetric Methods for the Separate Determination of Tyrosine, Tryptophane, and Cystine in Proteins, *J. Biol. Chem.*, 51: 421–434 (1922).

[58] May, C. E., and Rose, E. R., The Tryptophane Content of Some Proteins, *J. Biol. Chem.*, 54: 213–216 (1922).

[59] Patton, A. R., The Determination of Glycine in Proteins, *J. Biol. Chem.*, 108: 267–272 (1935).

[60] Bergmann, M., and Fox, S. W., Complex Salts of Amino Acids and Peptides. I. Metal Complex Salts of Glycine and Their Specificity, *J. Biol. Chem.*, 109: 317–324 (1935).

Reinecke's salt, $[Cr(CNS)_4(NH_3)_2]NH_4 \cdot H_2O$, precipitates secondary and tertiary amines in the form of very insoluble complexes and has been used for the separation of both proline and oxyproline from protein hydrolysates. Bergmann [61] prepared a compound analogous to the Reinecke complex in which aniline is substituted for the ammonium radicals. The new complex acid is tetrathiocyanatodianilidochromiato acid, $[Cr(CNS)_4(C_6H_5 \cdot NH_2)_2]H$, which he refers to as "rhodanilic acid." This acid forms an insoluble precipitate with proline but not with oxyproline. Accordingly proline may be precipitated and estimated as the rhodanilate, and from the filtrate oxyproline may be precipitated and estimated with Reinecke's salt.

13. *Other Methods.*—The above methods represent those usually employed for the isolation or determination of individual amino acids. A number of others have been proposed from time to time but have not been widely adopted. Perhaps the only one which needs special mention is that of Rapoport,[62] who has proposed that serine and glycine be determined together by treating the hydrolysate with nitrous acid, glycine going to glycolic acid, and serine to glyceric acid. The hydrolysate is then oxidized with alkaline permanganate, the glycolic acid and glyceric acid being converted to oxalic acid. The oxalic acid is precipitated as calcium oxalate and determined, after filtering and washing, with acid permanganate titration. Serine and glycine are the only monoaminomonocarboxylic acids which yield oxalic acid under this procedure.

Block [63] has recently prepared a critically annotated laboratory manual in which he outlines approved and tested methods for the quantitative or semi-quantitative determination of the various amino acids. The methods outlined in this manual are in a large measure those which have been thoroughly tested in the laboratories of Yale University and of the Connecticut Agricultural Experiment Station. This manual should be of great assistance to the biochemist interested in the problems of protein analysis.

[61] Bergmann, M., Complex Salts of Amino Acids and Peptides. II. Determination of *l*-Proline with the Aid of Rhodanilic Acid. The Structure of Gelatin, *J. Biol. Chem.*, 110: 471–479 (1935).

[62] Rapoport, S., Über eine Bestimmung der Summe von Glykokoll und Serin, *Biochem. Z.*, 281: 30–36 (1935).

[63] Block, R. J., The Determination of the Amino Acids, Burgess Publishing Company, Minneapolis, Minnesota (1938).

CHAPTER XV

PROTEIN CLASSIFICATION

Inasmuch as the exact chemical composition of nearly all the proteins is as yet unknown, it has been found convenient to divide them into classes, the basis of classification being chemical so far as possible, and where a chemical classification is not possible, solubility (or probably better, peptization, *vide infra*) has been made the basis. Two general classifications have been proposed, one by a joint committee of the American Physiological Society and the American Society of Biological Chemists, the other by the English Biochemical Society. These are referred to as the American classification and the English classification.

THE AMERICAN CLASSIFICATION

In 1908, the committees appointed by the American Physiological Society and the American Society of Biological Chemists recommended the classification [1] which is essentially reproduced below. In a few instances it has seemed wise to change the wording of their definitions slightly or to include additional comments.

I. The Simple Proteins.—These are the naturally occurring proteins which on being treated with enzymes or acids are hydrolyzed only into α-amino acids or their derivatives. They differ from the conjugated proteins in that the latter are not only hydrolyzed into amino acids but also yield other non-protein substances. Within the group of the simple proteins a number of subdivisions may be recognized, largely on the basis of solubility or other properties.

A. The Albumins.—The albumins are soluble in water and in dilute salt solutions and are coagulable by heat. Typical examples are ovalbumin from egg white, lactalbumin from milk, and vegetable albumins such as the leucosin from wheat. (As a matter of fact, some of the albumins which have been thoroughly studied, notably egg albumin and serum albumin, contain carbohydrate residues and should accordingly be classified strictly with the conjugated proteins under the subclass of "glycoproteins." Both the American and English classifications, however, list them as the first subclass of the simple proteins. This fact is an illustration of the difficulties of exact protein classification.)

[1] Joint Recommendations of the Physiological and Biochemical Committees on Protein Nomenclature, *J. Biol. Chem.*, 4: xlviii-li (1908).

B. The Globulins.—The globulins are simple proteins, insoluble in pure water, but soluble in dilute neutral solutions of the salts of strong bases and acids. Typical examples are the ovoglobulin of egg yolk, the myosin of muscle, and edestin from hempseed. Typical globulins have been isolated from a great many vegetable seeds.

C. The Glutelins.—These are simple proteins, insoluble in all neutral solvents, but readily soluble in *very* dilute acids and alkalies. Examples are the glutenin from wheat, oryzein of rice, etc.

D. The Prolamines or Alcohol-soluble Proteins.—This class is defined as being insoluble in water, absolute alcohol, or other neutral solvents, but soluble in relatively strong alcohol (70–80 per cent). Typical examples are zein from corn, gliadin from wheat, hordein from barley; with a single exception (the alcohol-soluble protein from milk, isolated by Osborne), the prolamines are confined to the seeds of the cereal grains.

E. The Albuminoids.—These are defined as simple proteins which possess essentially the same chemical composition as other proteins, but are characterized by a great insolubility in all neutral solvents. They are, in general, insoluble in dilute acid, alkali, water, or salt solutions. This group is in reality a heterogeneous group, containing various proteins of widely different physical properties. Apparently all the proteins which do not fit definitely into some other class are grouped with the albuminoids. Examples are the keratin from horn, hide, hoof, hair, and feathers of animals, elastin from ligaments, collagen from hide and tendons, and gelatin from hide, hoofs, bones, etc. Gelatin, though classed as an albuminoid, does not agree with the definition noted above. It is more properly a protein derived from collagen, which fact probably accounts for its inclusion in the group.

F. The Histones.—Histones are soluble in water, insoluble in very dilute ammonia, not coagulable by heat, easily soluble in very dilute acids or solutions of the fixed alkalies, and on acid hydrolysis yield a large number of amino acids, among which the basic amino acids predominate. As a general rule, histones form precipitates with solutions of other proteins. These precipitates are probably coacervates formed under conditions where the histones are positively charged but the other proteins are on the negative side of their isoelectric point. A similar precipitate which appears to be quite characteristic of the histones is formed with sodium alizarine sulfonate and is used for the characterization of the histones. Histones are basic proteins, their basicity being intermediate between the protamines and the more common proteins. Typical examples are the globin from hemoglobin and the histones from birds' corpuscles and from the thymus gland.

G. The Protamines.—These are the simplest natural proteins. In reality they may be regarded as simply large polypeptides. Waldschmidt-Leitz, in his recent study of certain of these proteins, con-

cludes that they contain only 14 or 15 peptide linkages and that probably their molecular weight does not exceed 3,000. They are highly basic, soluble in water, soluble in ammonia, form true salts with mineral acids, many of the salts being crystalline. They are not coagulable by heat, and yield on hydrolysis relatively few amino acids with the diamino acids greatly predominating. They have the property of precipitating other proteins from aqueous solution again probably as coacervates. They form coacervates with nucleic acid, with arabic acid, and with other negatively charged lyophilic colloids. They are typically found in the ripe sperm cells and have been largely isolated from fish sperm. Examples are salmine, containing 88 per cent of arginine, from salmon sperm, sturine from sturgeon sperm, etc.

II. CONJUGATED PROTEINS.—These are compounds of simple proteins with some other non-protein group, the union with the non-protein molecule being otherwise than as a salt.

A. Nucleoproteins.—These are compounds of one or more protein molecules with nucleic acid. They are probably coacervate systems. They are the proteins of the cell nuclei and apparently make up in a large measure the substance of the chromatin. Examples are nuclein and nucleohistone from tissues rich in cells, such as glandular tissues, yeast, etc.

B. Glycoproteins or Glucoproteins.—These are proteins in which the additional group is a carbohydrate radical other than in the form of that contained in nucleic acid. The mucus-yielding proteins of tissues are particularly rich in glycoproteins. They possibly serve as a cementing substance for holding together the fibers in tendons and ligaments. The mucin which is secreted by the snail or by the salivary glands, the protein making up the great majority of the structure of the jellyfish, and the proteins forming the jelly surrounding fish and amphibian eggs are typical examples of these proteins.

C. Phosphoproteins.—The prosthenic group in these proteins is *o-phosphoric* acid esterified either as the monoester or as the diester with the —OH group of the hydroxyamino acids, particularly with serine. Typical examples are casein from milk, and vitellin from egg yolk.

D. Chromoproteins.—This group is referred to in the original classification as the *hemoglobins*. It should, however, be extended to include other proteins than the hemoglobins. The conjugated group is colored and may be hematin as in hemoglobin, cyanin as in hemocyanin, or a group of unknown nature, such as occurs in the colored proteins of certain seaweeds where the proteins have been named phycoerythrin and phycocyan. Chromoproteins likewise occur in certain pigmented animal fibers, such as black wool and hair. The colored group is melanin. Visual purple of the retina of the eye is a chromoprotein in which the colored group is a carotenoid.

E. Lecithoproteins.—These are the proteins of the cytoplasm and of the cell membrane area. Again, they are probably coacervate systems. The conjugated group is lecithin or a phospholipid. Lung tissue is rich in such proteins.

F. Lipoproteins.—This group was added by A. P. Mathews, the conjugated group being one of the higher fatty acids. Such compounds are so easily prepared artificially that their natural occurrence is deemed probable.

III. Derived Proteins.—This group includes the various decomposition products of the naturally occurring proteins which have been produced by the action of reagents or enzymes or physical agents, such as heat, hydrogen-ion concentration, etc. It also includes the artificially synthesized compounds. It is divided into various groups according to solubility, and to a lesser extent according to the degree of complexity.

A. Primary Protein Derivatives.

1. *Coagulated Proteins.*—These are insoluble protein products produced from natural proteins by the action of heat, alcohol, or by some similar method.

2. *Proteans.*—These are the initial product of the action of very dilute acids or, in some instances, water or enzymes, upon certain globulins. This form of derived protein is particularly characteristic of the globulins and differs physically from the globulins by a loss of solubility in dilute salt solutions. Except for the fact that they have been produced by laboratory technic from "globulin," they have all the characteristics of the naturally occurring glutelins. Examples are edestan from edestin, myosan from myosin, etc.

3. *Metaproteins.*—These are produced by the further action of acids and alkalies upon proteins. They are, as a rule, characterized by being soluble in very weak acids or alkalies but by being insoluble in neutral solutions. Examples are acid metaprotein (acid albuminate) and alkali metaprotein (alkali albuminate).

B. Secondary Protein Derivatives.

1. *Proteoses.*—The partial hydrolytic decomposition products of proteins. These are soluble in water, non-coagulable by heat, and *precipitated* by saturating their solutions with ammonium sulfate.

2. *Peptones.*—The partial hydrolytic decomposition products of proteins. They are soluble in water, non-coagulable by heat, and *not precipitated* by saturating the solutions with ammonium sulfate.

3. *Peptides.*—These are definitely characterized compounds of two or more amino acids, the carboxyl group of one being united with the amino group of the other, with the elimination of a molecule of water.

They are not heat-coagulable; they may or may not give the biuret reaction; they may be either of natural origin or synthetic.

4. *Diketopiperazines.*—These are cyclic anhydrides of two amino acids. They may be regarded as the anhydride of a dipeptide.

THE ENGLISH CLASSIFICATION

The English classification differs from the American classification only in minor details.

I. Simple Proteins
- A. Protamines
- B. Histones
- C. Globulins
- D. Albumins
- E. Glutelins
- F. Gliadins (prolamines) (soluble in 80 per cent alcohol; insoluble in water)
- G. Scleroproteins (forming the skeletal structure of animals)
- H. Phosphoproteins

II. Conjugated Proteins
- A. Chromoproteins
- B. Nucleoproteins
- C. Glucoproteins

III. Hydrolyzed Proteins
- A. Metaproteins
- B. Albuminoses or proteoses
- C. Peptones
- D. Polypeptides

The definitions adopted for these groups are essentially those already given in the American classification. It will be noticed that the English classification places the phosphoproteins among the simple proteins.

THE CRITERIA OF PROTEIN CLASSIFICATION

A casual glance at the above systems of classification indicates definitely that the major distinction lies in physical properties. *Solubility* and *precipitability* have been emphasized at a number of points. Since the above classifications were adopted a great deal of attention has been given to the physicochemical properties of protein systems. No one appears to have definitely challenged the generally accepted systems of protein classification prior to the paper of Gortner, Hoffman,

and Sinclair,[2] although many workers have pointed out the fact that changes in solubility could be brought about by various reagents.

Hoffman and Gortner,[3] in an attempt to isolate a quantity of the various proteins which had been reported to occur in wheat flour, observed that extracting the flour with 5 per cent potassium sulfate solution and with 10 per cent sodium chloride solution did not yield similar fractions, but that, instead, the amount and character of the protein material dissolved by these two reagents were markedly different. These differences were especially noticeable in the globulin fraction. According to the definition of globulin, the two solutions should have yielded identical fractions.

Accordingly, Gortner, Hoffman, and Sinclair definitely raised the question which is ignored in the definition of globulins, *i.e.*, "simple proteins, heat-coagulable, insoluble in water, but soluble in dilute solutions of the salts of strong acids and bases," and they ask the question, *"What salts, and what concentrations?"* They undertook an extensive study of this question, utilizing wheat flour as the biological material and studying the behavior of 22 different salts, most of them in three or more concentrations.

In this study they used 12 different wheat flours, practically all of which were extracted with the various concentrations of the various salt solutions. Table LXII shows the *average* percentages of protein extracted from this series of wheat flours by the various concentrations of the different salt solutions. In order to be certain that the effects observed were due to the salt solutions and not to variations in hydrogen-ion concentration, the 1.0 *N* solutions of the potassium halides were carefully adjusted to the same hydrogen-ion concentration. A lyotropic series of $KF < KCl < KBr < KI$ was found, with extreme ranges in the percentage of total protein extracted of from 13.07 per cent to 63.89 per cent. *In this instance, at least, the degree of peptization can be due only to a specific influence of the anions which are present in equivalent concentrations.* It seemed pertinent, therefore, definitely to raise the question, "What is a globulin?"

The authors point out that, in the light of these results, it should be obvious that a definition which is based upon solubility, *i.e.*, peptization, in a dilute salt solution is so ambiguous as to be absolutely meaningless. They further point out that all the various protein fractions which have been considered to be present in wheat flour and which

[2] Gortner, R. A., Hoffman, W. F., and Sinclair, W. B., Physico-Chemical Studies on Proteins. III. Proteins and the Lyotropic Series, Colloid Symposium Monograph, Vol. V, pp. 179–198 (1928); *cf.* also *Kolloid. Z.*, 44: 97–108 (1928); *Cereal Chem.*, 6: 1–17 (1929).

[3] Hoffman, W. F., and Gortner, R. A., The Preparation and Analysis of the Various Proteins of Wheat Flour with Special Reference to the Globulin, Albumin, and Proteose Fractions, *Cereal Chem.*, 4: 221–229 (1927).

TABLE LXII

AVERAGE PERCENTAGE OF TOTAL PROTEIN* EXTRACTED FROM 12 WHEAT FLOURS BY VARIOUS CONCENTRATIONS OF SALT SOLUTIONS

Salt	Concentration of Salt		
	0.5 *N*	1.0 *N*	2.0 *N*
Li acetate	25.01	22.32	
LiCl	30.08	29.32	28.60
NaCl	23.16	21.68	18.97
Na_2SO_4	20.00	18.20	17.30
Na_3 citrate	25.07	25.13	24.52
Na_2HPO_4†	27.44	25.62	25.32
KF	.....	13.07	11.93
KCl	24.62	22.77	20.01
KBr	38.77	37.22	34.91
KI	.....	63.89	
K_2SO_4	19.68	18.59	
K_2 tartrate	26.35	24.12	
K_2CrO_4	26.92	.....	
$MgCl_2$	33.01	34.99	38.35
$MgBr_2$	30.17	40.11	54.38
$MgSO_4$	26.18	26.35	25.69
$CaCl_2$	34.14	36.60	32.25
$CaBr_2$	33.90	43.27	53.68
$SrCl_2$	32.59	34.50	36.47
$BaCl_2$	27.29	33.42	29.24
$ZnSO_4$	.....	16.71	
$Al_2(SO_4)_3$	.....	16.90	

* Each figure represents the average of at least 24 separate nitrogen determinations.
† Considered as a divalent salt.

are regarded as fixed entities can be isolated in a constant proportion, provided that a prescribed routine procedure for protein isolation is followed. If, however, that prescribed routine procedure is altered somewhat, different results are obtained. This is to be expected if one considers that *the system in question is a colloid system and that solubility is synonymous with peptization.* On the other hand, it likewise means that the fractions which are isolated by a prescribed technic are not necessarily chemical entities but may represent only a peptized fraction.

They further point out that we know far too little about the physicochemical factors underlying protein peptization to assign a cause for the variations noted in Table LXII. The cause is probably complex, involving the nature of amino-acid linkages, secondary valence, polar groups, degree of hydration, specific ionic effects, electrokinetic forces, etc., and until at least some of these have been evaluated, it is useless to speculate as to the causes involved. On the other

hand, the mere fact that one can repeatedly isolate a given quantity of protein from biological material, using a rigidly standardized technic, is no valid proof that the material which is isolated constitutes a chemical entity.

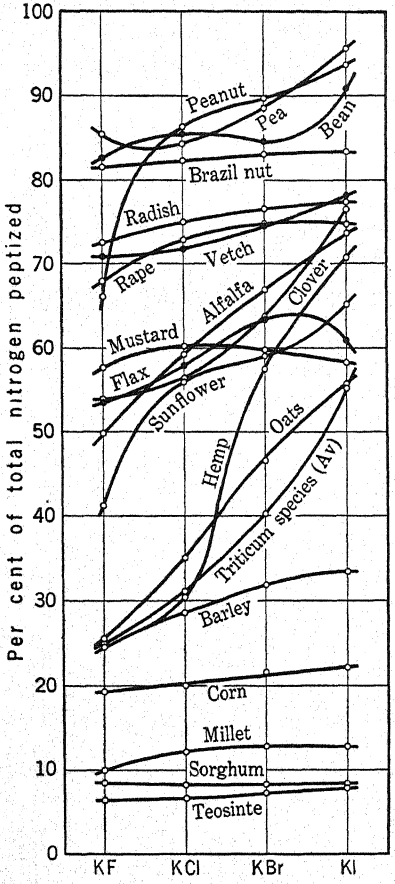


FIG. 121.—Showing the peptization behavior of 0.5 *M* solutions of the potassium halides on the protein complex present in various seeds and grains. (Data of Staker and Gortner.)

The term globulin is assigned to a group of proteins separable from other protein fractions of tissues by a purely arbitrary procedure. The globulins have been separated into euglobulins and pseudoglobulins on an equally arbitrary basis. When a globulin changes its solubility and is transformed into a protean, such as myosin to myosan, it is classified as a "derived protein," although nothing is known of the chemical change which has come about, and although the protean has practically all the properties of the class of simple proteins known as glutelins. No one can say that the glutelins are not proteans which have undergone the globulin → protean transformation in nature.

In an attempt to ascertain how generally the phenomena of peptization might be applied to the problems of protein behavior, Staker and Gortner,[4] using 0.5 *M* solutions of KF, KCl, KBr, KI, and K_2SO_4, studied most of the seeds and grains which had formed the basis of Osborne's work on the vegetable proteins. Figure 121 shows a graph of certain of the data obtained. A study of the ratios of albumin to globulin using the various technics indicated that *the albumin: globulin ratio varied widely depending upon the particular technic which was employed, the variations in some in-*

[4] Staker, E. V., and Gortner, R. A., Physico-Chemical Studies on Proteins. V. A Comparative Study of the Peptization of the Protein Complex in Various Seeds and Grains, *J. Phys. Chem.*, 35: 1565–1605 (1931).

stances being as much as 700 per cent. In view of this observation, it is not surprising to find that one worker, using one technic, reports a high content of albumin and a low content of globulin, and another worker, using a different technic, reports exactly the opposite.

At about the same time that the above studies were in progress, the workers at the Carlsberg laboratories [5] were studying the solubility behavior of various proteins. These studies culminated in Sörensen's [6] theory of "reversible-dissociable component systems." The gist of this theory can be expressed in Sörensen's own words: "Soluble proteins consist of a series of complexes or components, reversibly combined, which makes their constitution expressible by the ordinary formula $A_xB_yC_z$ . . . *A, B, C* and so on each marking complete complexes, mainly polypeptides, yet in some cases also containing other groups, for example phosphorous ones, whereas the affixed indices *x, y, z* and so on mark the amount to which the indicated complex is present in the entire component system. Within each complex all the atoms and atom groups are linked together by main-valences, whereas the various complexes in the whole component system are comparatively loosely and reversibly knit together by means of the residual valences which each component must be assumed to possess, and the strength and nature of which must depend on the chemical composition of the component in question as well as on its physical properties, above all on its dimensions and the resulting shape and surface. But all things considered, the linkage between the components must be supposed to be comparatively slight and of such a nature that alterations in the composition of the solution (salt content, hydrogen-ion activity, alcohol content, temperature) may give rise to reversible dissociations of the involved component systems and interchange of components between the same. When these alterations in the composition of the solution are so suited as to render possible in sufficient quantities the formation of a component system insoluble or sparingly soluble under the new conditions, such a system will be formed and precipitated. In good accord with this is the fact that through suitable proceedings it has been possible to effect a reversible fractionation in the case of all hitherto investigated proteins. In the main the fractions gained possess

[5] Sörensen, S. P. L., Studies on Proteins. VIII. On the Solubility of the Serum Globulins, *Compt. rend. trav. lab. Carlsberg*, Vol. 15, No. 11 (1925); Linderström-Lang, K., and Kodama, S., Studies on Casein. I. On the Solubility of Casein in Hydrochloric Acid, *ibid.*, Vol. 16, No. 1 (1925); Linderström-Lang, K., Studies on Casein. III. On the Fractionation of Casein, *ibid.*, Vol. 17, No. 9 (1929); Sörensen, S. P. L., and Sládek, I., On Wo. Ostwald's "Bodenkörper-Regel" and the Solubility of Casein in Sodium Hydroxide, *ibid.*, Vol. 17, No. 14 (1929); and Haugaard, G., and Johnson, A. H., On the Fractionation of Gliadin, *ibid.*, Vol. 18, No. 2 (1930).

[6] Sörensen, S. P. L., The Constitution of Soluble Proteins, *Compt. rend. trav. lab. Carlsberg*, Vol. 18, No. 5 (1930).

indeed the properties of the initial material, yet both the physical properties and the chemical composition are more or less modified from fraction to fraction because of their varying contents of the different components."

Sörensen's theory differs from the peptization theory of Gortner *et al.*, primarily in the fact that he regards solubility differences as being due to the heterogeneous nature of the protein micelle and dependent upon specific chemical configurations in the various peptide units, whereas the peptization theory lays emphasis upon physical surface forces and colloidal phenomena. Under Sörensen's theory a given protein aggregate can be fractionated into a series of fractions, each fraction possessing its own individual physical properties and characterized by its own specific chemical composition, the physical properties being a function of the chemical composition. A recombination of different fractions would then bring about a system possessing the same physical and chemical characteristics as were possessed by the original protein aggregate. In order to test whether or not a recombination of fractions was necessary or whether the original physical behavior of the protein aggregate was primarily determined by the colloidal state of the system, gliadin was repeatedly extracted [7] with molar KI solution until no more protein could be peptized from the residue. It was found that approximately 75 per cent of the gliadin was "dissolved" by the KI solution and approximately 25 per cent remained as a non-peptizable residue. On the basis of Sörensen's theory this should have resulted in a *chemical* fractionation of the original gliadin, and by a recombination of these fractions the properties of the original gliadin preparation should have been regained. However, the "soluble fraction" and the "insoluble fraction" were *separately* reworked (electrodialyzed, dissolved in alcohol, repeptized, redissolved, and reworked by the "standard method" for the preparation of gliadin) so as to secure dry white gliadin powder as in the original protein preparation. These two fractions were then subjected to peptization with KI as in the original preparation. *The peptization behavior of these separate fractions greatly resembled the peptization behavior of the initial protein preparation.* Approximately 75 per cent of the "KI-soluble" fraction was peptized by the second series of KI extractions, and approximately the same percentage of the reworked "KI-insoluble" fraction was similarly peptized. Here the properties of the original preparation were essentially regained not by a *recombination* of polypeptide fractions as Sörensen's theory requires but by simply reworking either the "soluble" or the "insoluble" fraction by as nearly as possible the same standard technic as was used to prepare the

[7] Sinclair, W. B., and Gortner, R. A., Physico-Chemical Studies on Proteins. VII. The Peptization of Gliadin by Solutions of Inorganic Salts, *Cereal Chem.*, 10: 171–188 (1933).

original sample of gliadin. The dry purified protein had been brought back to approximately the same *physical* state, and the subsequent peptization behavior of the colloidal aggregates in the dry protein gel reflected the similarity in physical state. The two fractions of gliadin noted above could not be differentiated from each other by any *chemical* analyses which were applied.

There is no question but that in some instances the individual fractions of a "protein aggregate" do differ from one another in chemical composition. Workers at the Carlsberg laboratories have shown that to be true in certain of their studies. Blish and Sandstedt [8] showed it for glutenin. Lustig,[9] using non-standard technic, isolated 4 "euglobulins," 4 "pseudoglobulins," and 3 "albumins" from blood serum and showed that these differ from one another in "solubility" and in chemical composition. Block [10] from an independent series of studies on blood serum reaches the following conclusions:

"Serum does not contain several independent proteins. The fractions isolated by physico-chemical methods are not pre-existent in the serum but result from the technic employed."

"The albumins and globulins obtained from cattle serum by precipitation with various neutral salts are not of constant basic amino acid composition but are simply artificial products produced by the reagents employed in their preparation."

"A better characterization of serum protein could be effected by a study of the whole protein rather than by attempting to isolate any 'pure' preparation even though it might be crystalline."

"Proteins isolated from living tissue are not necessarily present as such in that tissue during life."

Other workers have reached similar conclusions. Perhaps it is sufficient to cite two additional studies. McCalla and Rose [11] dispersed wheat gluten in a solution of sodium salicylate and precipitated a series of fractions from this solution by the gradual addition of magnesium sulfate. The various fractions were analyzed for total nitrogen, amide nitrogen, and arginine nitrogen and were found to vary systematically from fraction to fraction, the amide nitrogen increasing from the first to the seventh fraction, the arginine nitrogen at the same time decreasing. They reached the conclusion that there is no evidence for the existence of "gliadin" or "glutenin" as independent proteins in

[8] Blish, M. J., and Sandstedt, R. M., Concerning the Nature of the Protein Extracted from Wheat Flour by Hot Alcohol, *Cereal Chem.*, 6: 494–503 (1929).

[9] Lustig, B., Zur Kenntnis der Unterfraktionen der Globuline und Albumine im Serum, I–IV, *Biochem. Z.*, 225: 247–263 (1930); *ibid.*, 231: 39–44; *ibid.*, 231: 472–492; *ibid.*, 238: 307–320 (1931).

[10] Block, R. J., The Basic Amino Acids of the Serum Proteins, *J. Biol. Chem.*, 103: 261–267 (1933); *cf.* also *J. Biol. Chem.*, 104: 343–346 and 347–350 (1934).

[11] McCalla, A. G., and Rose, R. C., Fractionation of Gluten Dispersed in Sodium Salicylate Solution, *Canadian J. Research*, 12: 346–356 (1935).

wheat gluten and that "the terms 'glutenin' and 'gliadin' should be discarded. . . . Gluten is a single protein complex which may be separated into a great many fractions which differ progressively and systematically in both physical and chemical properties." Rich,[12] from an independent study of wheat protein, reaches a similar conclusion: "By arbitrarily choosing the methods of isolation, five protein fractions may be obtained. If, however, the dispersing agent or the method of isolation is changed, even slightly, there is no established limit as to the *number, quantities,* and *character* of the protein fractions that can be isolated." In the two succeeding chapters we shall have occasion to discuss further evidence bearing on this problem of protein homogeneity, protein peptization, and the reactions characteristic of protein systems. The American system of protein classification will be employed in the later discussions, but it is desirable for the student to remember that the "albumins," "globulins," etc., which will be discussed are probably laboratory artifacts arising from a protein complex and that *the properties of the protein complex as it exists in the cells and tissues of the living organism are the all-important determiners of cell behavior and the properties of "albumins" and "globulins" as we study them in the laboratory may bear no direct relationships to the properties of proteins as they exist in living tissues.* The research worker should recognize that solubility is nothing more nor less than peptization and that the proteins must be considered not alone as complex organic compounds but likewise as colloid micelles, subject to all the varied reactions of a lyophilic system. Only under such conditions will the study of protein classification and reactions characteristic of protein systems be advanced.

[12] Rich, C. E., Some Physico-Chemical Properties of Wheat Flour Proteins, *Cereal Chem.*, 13: 522–541 (1936).

CHAPTER XVI

PROTEIN STRUCTURE AND THE POSSIBILITIES OF PROTEIN ISOMERISM

We have already noted that the peptide linkage has been definitely proven to be a major linkage in proteins. We have also indicated that polypeptides which have been obtained by the partial hydrolysis of proteins have been artificially synthesized, so that their structure is known. More recently a number of workers have laid emphasis on the properties of other forms of linkage than that of the long-chain polypeptide type. We cannot discuss any of these viewpoints at length, but it seems worth while to indicate the trend of thought in this field.

THEORIES OF PROTEIN STRUCTURE.—Critical reviews [1] of the various hypotheses which have been proposed to account for protein structure are available. Although these are possibly ultraconservative, nevertheless they bring together a number of divergent views and compare them one with another.

1. *Kossel's "Protamine Nucleus" Hypothesis.*—Kossel [2] postulated from his study of the diamino acids that the nucleus of all proteins would be found to be of the "protamine" type, *i.e.*, to be largely composed of either arginine, histidine, or lysine, or combinations of these three amino acids. Kossel's hypothesis was more or less ignored for a period of years. Larmour,[3] however, again called attention to this hypothesis in a statistical study of the relationship which exists between the total basic nitrogen and the arginine nitrogen or the lysine nitrogen of the various proteins, as determined by Van Slyke's method. From a statistical study of 214 analyses of proteins by Van Slyke's method, he finds a coefficient of correlation of $r = +0.794 \pm 0.017$ between the total basic nitrogen and the arginine nitrogen, and a coefficient of correlation between the total basic nitrogen and the deviation of the arginine nitrogen from its most probable value, the mean, of

[1] Vickery, H. B., and Osborne, T. B., A Review of Hypotheses of the Structure of Proteins, *Physiol. Rev.*, 8: 393–446 (1928); *cf.* also Vickery, H. B., Recent Contributions to the Theory of Protein Structure, *Yale J. Biol. Med.*, 4: 595–610 (1932).

[2] Kossel, A., Über die basischen Stoffe des Zellkerns, *Z. physiol. Chem.*, 22: 176–187 (1896).

[3] Larmour, R. K., Correlations between the Total Nitrogen of the Bases and Arginine Nitrogen and between Total Nitrogen of the Bases and Lysine Nitrogen, of Various Proteins, *Trans. Roy. Soc.*, Canada, Section V, 349–363 (1928).

$r = +0.211 \pm 0.044$. The coefficient of correlation between total basic nitrogen and lysine nitrogen for 213 cases was found to be $r = +0.548 \pm 0.032$, and the coefficient of correlation between total basic nitrogen and the deviation of lysine from its most probable value, the mean, was found to be $r = +0.03 \pm 0.046$. Larmour believes that the high correlations in the case of arginine are evidence in support of Kossel's hypothesis that arginine is the nucleus of the protein molecule and states, "It seems highly probable that in all proteins there is a direct and marked relationship between the arginine and the total basic nitrogen."

Block,[4] in a series of papers, finds that, although proteins may be fractionated into fractions which differ both in physical and chemical properties, nevertheless there is a strong tendency for the ratios between arginine, histidine, and lysine to remain relatively constant, and he regards this as evidence "for a basic amino acid 'anlage'" as existing in the proteins. Alcock[5] takes a somewhat similar view of a "urprotein" in which arginine, histidine, lysine, cystine, and tryptophane play a greater role than the other amino acids, and in a number of other papers emphasis has been laid on the possible role which the diamino acids play in determining the basic structure of proteins.

2. *Abderhalden's Anhydride Theory.*—As early as 1902 Fischer noted that leucinimide, a substituted diketopiperazine, could be separated from a tryptic-peptic digestion of hemoglobin, and he added that it unquestionably existed preformed in the protein molecule. Some twenty years later, Abderhalden began an extensive series of investigations on the structure of proteins, and as a result of these studies he concludes that proteins are to a very considerable extent built up of cyclic substituted diketopiperazine rings held together by the force of latent valence. The literature which Abderhalden and his students have presented is altogether too voluminous to be cited in detail. Most of the papers[6] will be found in the *Zeitschrift für physiologische Chemie.*

From a study of diketopiperazines which have been prepared synthetically, Abderhalden finds that their behavior toward certain chemical reagents is much more nearly like the behavior of proteins toward these reagents than is the behavior of straight-chain polypeptides. He

[4] Block, R. J., papers on serum proteins (*loc. cit.*) *cf.* also *J. Biol. Chem.*, 93: 113–117 (1931); 104: 339–341 (1934); 105: 663–666 (1934).

[5] Alcock, R. S., The Synthesis of Proteins in Vivo, *Physiol. Rev.*, 16: 1–18 (1936).

[6] For summaries see: Abderhalden, E., Das Eiweiss als eine Zusammenfassung assoziierter, Anhydride enthaltender Elementarkomplexe, *Naturwissenschaften*, 12: 716–720 (1924); Über die Struktur der Proteine, *Z. physiol. Chem.*, 128: 119–128 (1923); Abderhalden, E., and Schwab, E., Über die Anhydridstruktur des Seidenfibroins, *ibid.*, 139: 169–180 (1924).

developed certain color reactions, notably that given by picric acid and alkali and that given by *m*-dinitrobenzene in alkaline solution, which are characteristic of diketopiperazines, and finds that proteins and peptones of high molecular weight give the characteristic color reaction, whereas polypeptides do not. He also separated from the partial hydrolytic products of proteins a number of diketopiperazines.

Abderhalden [7] points out that the presence of an anhydride structure possessing keto $\rightleftharpoons$ enol isomerism may well be responsible for the extreme lability of certain proteins. He notes that, even in Fischer's octadecapeptide or in his own nonadecapeptide, the polypeptide showed a great stability and was not readily altered or affected by chemical reagents which would not hydrolyze it, and he contrasts this behavior with the behavior of vital proteins. Certain of his comments are so important to the student of vital phenomena that it seems worth while to append them in the form of a rather free translation. Thus he states, "Each conception in regard to particular structural relations in proteins, and especially of those which are concerned in living processes, must take into consideration all their reactions, their ready transformation from the natural to the denatured condition, and their greater or less lability. It is certain that proteins in protoplasm have properties of which we are at present entirely unaware. We study proteins almost invariably in a more or a less changed condition. On the one hand, we have proteins which in a certain sense are denatured in the organism, and outside of the organism undergo further changes. Thus, for example, we see the silk threads, the web of the spider, the byssus of certain mussels, etc., which are extruded as a liquid, changing to an inert solid. From a very labile form they are transformed into a very stable structure. In living processes we deal with the very reactive cell proteins which regulate in a fine degree the reactions of the cell, but when death ensues, the cell proteins coagulate and lose in a large measure their characteristic physical behavior."

Brill [8] investigated the X-ray diffraction pattern of certain proteins, notably silk fibroin, and reached the conclusion that the basic structure consists of four molecules of glycylglycine combined in a ring form, probably as an anhydride.

Such a ring structure has been cited as evidence in favor of Abderhalden's diketopiperazine theory, but other workers have interpreted the X-ray diagrams as evidence of a series of spirals in the polypeptide chain forming a series of nearly closed loops which might give rise to a diffraction pattern similar to that of the kiketopiperazine structure. Such a series of loops is shown in Fig. 122. In this figure the spacing

[7] Abderhalden, E., and Komm, E., Über die Anhydridstruktur der Proteine, *Z. physiol. Chem.*, 139: 181–204 (1924).

[8] Brill, R., Über Seidenfibroin, I., *Ann.*, 434: 204–217 (1923).

of 5.15 Å, as observed by Astbury and Woods [9] in an X-ray study of wool fibers, represents the normal spacing of the unstretched fiber. The X-ray diagrams indicate that the six-membered loops all lie in the

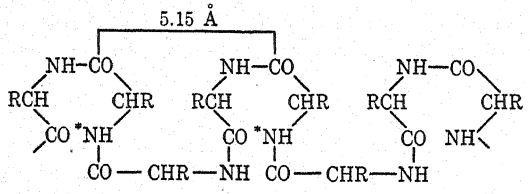


FIG. 122.—A possible space arrangement of amino acid units in a peptide chain.

same plane, whereas the seven-membered loops are skewed at an angle to the plane of the six-membered loops. Since the —NH and the —CO groups lie near to each other at the opening of the six-membered loops, there must be attraction between these electropositive and electronegative groups, so that they are either actually linked into diketopiperazine rings or hydrogen bridges are formed which would have a high degree of stability. It may be that the diketopiperazine structure does not exist preformed in the protein but that the action of reagents in hydrolyzing the protein causes a condensation between the —NH and —CO groups of the six-membered loops and thus gives rise to diketopiperazines in the hydrolysate.

While the X-ray diagrams of proteins are under consideration, mention should be made of the later observation of Brill [10] that the fluid contents of the spinning gland of the spider do not yield an X-ray diffraction pattern, but when the fluid contents are dried the pattern appears. This emphasizes Abderhalden's comments given above.

Some observations of Levene and Bass [11] are pertinent to the question of polypeptide versus diketopiperazine structure. They note that polypeptides hydrolyze slowly and racemize slowly at low alkali concentration and that they hydrolyze slowly but racemize more rapidly at high (1.0 *N*) alkali concentration. Diketopiperazines, on the other hand, racemize rapidly at low alkali concentrations and at high alkali concentrations hydrolyze so rapidly that the amino acids of which they are composed are not racemized. Casein does not act like either polypeptides or the diketopiperazines. Levene and Bass ran the racemiza-

[9] Astbury, W. T., and Woods, H. J., The X-Ray Interpretation of the Structure and Elastic Properties of Hair Keratin, *Nature*, 126: 913–914 (1930); The Molecular Weights of Proteins, *Nature*, 127: 663–665 (1931).

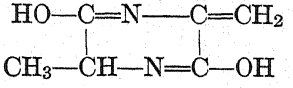
[10] Brill, R., Zur Kenntnis des Seidenfibroins, *Naturwissenschaften*, 18: 622 (1930).

[11] Levene, P. A., and Bass, L. W., Studies on Racemization VII. The Action of Alkali on Casein, *J. Biol. Chem.*, 78: 145–157 (1928); VIII. The Action of Alkali on Proteins: Racemization and Hydrolysis, *ibid.*, 82: 171–190 (1929).

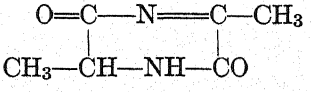
tion curves and the amino nitrogen : total nitrogen ratios and found that the amino acids of casein were completely racemized in 1.0 *N* alkali at 125°. They conclude that either there are no diketopiperazines in the casein molecule or that, if diketopiperazines are present, the linkage is much more stable than those diketopiperazine linkages that have been studied in the laboratory.

3. *Bergmann's Hypothesis.*—Bergmann postulates a protein structure as being composed of piperazine derivatives held together by forces of latent valence. His theory is very similar to that of Abderhalden, with the exception that Bergmann [12] has been particularly interested in the reactions of hydroxyamino acids and their effect upon the ring structure.

He suggests that a dipeptide, such as alanyl serine, may lose water to form the usual 2-methyl-5-hydroxymethyl-3-6-diketopiperazine and that this may further lose water, the 5-hydroxymethyl group going to a methylene group. The 2-methyl-5-methylene-3.6-diketopiperazine then rearranges to an "iso" form:



This "iso" form forms a disodium salt and a diacetate and is capable of rearrangement to an "allo" form:



This "allo" form polymerizes so that in the presence of water it yields a colloidal hydrosol which adsorbs tannin and a number of dyestuffs, and the polymerized product in many of its reactions resembles the reactions of protein.

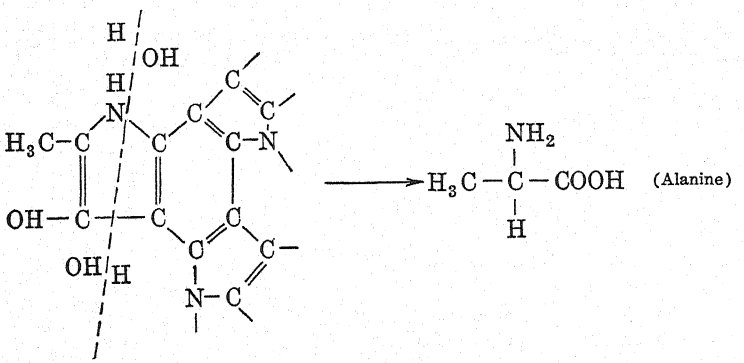
Bergmann points out that none of the simple polypeptides or the simple diketopiperazines adsorb tannin in appreciable amount, whereas his polymerized "allo" form is strikingly like the proteins in that regard. Furthermore, when the "allo" form was subjected to mild hydrolysis, he isolated not the original dipeptide but rather a tetrapeptide which led him to suggest that the isolation of tetrapeptides from proteins is not necessarily proof that the tetrapeptide existed preformed in the protein molecule but may have been derived from some cyclic

[12] Bergmann, M., Über neuere Proteinchemie, *Naturwissenschaften*, 12: 1155–1161 (1924); Über den hochmolekularen Zustand der Proteine und die Synthese proteinähnlicher Piperazin-Abkömmlinge, *ibid.*, 13: 1045–1050 (1925). *Cf.* also numerous papers in the *Annalen* and in *Zeitschrift für physiologische Chemie*, and especially Synthesis and Degradation of Proteins in the Laboratory and in Metabolism, *Science*, 79: 439–445 (1934).

structure analogous to his polymerized "allo" form. He suggests that the "allo" form polymerizes through secondary valence forces which are resistant toward water or ordinary aqueous solutions.

Bergmann admits that no definite proof exists for the presence of such compounds in the protein molecule but believes that the hydroxy-amino acids would undergo similar reactions.

4. *Troensegaard's Pyrrole Hypothesis.*—Troensegaard [13] developed a theory of protein structure based largely upon a study of completely acetylated proteins and the hydrolytic products of such proteins. He postulates the presence of heterocyclic rings, largely of the pyrrole type, as indicated in the following diagram:



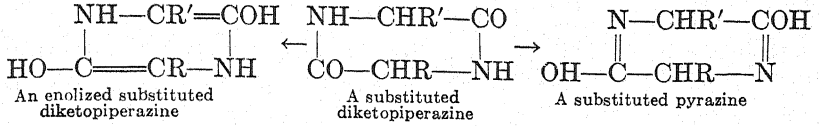
Hydrolysis is postulated as occurring along the dotted line, liberating alanine as one of the hydrolytic products. As Vickery and Osborne point out, such a structure as Troensegaard postulates would not appear to be the normal structure characteristic of proteins for two reasons: (1) the double bond in the condensed alanine structure should, when hydrolyzed according to Troensegaard's scheme, yield a racemic mixture of amino acids rather than a single optically active acid; and (2) the proposed ring system contains an arrangement of six carbons identical with the arrangement of carbons in the benzene ring. Such a structure should be very stable when submitted to acid hydrolysis.

5. *Karrer's Hypothesis.*—Karrer [14] calls attention to the possibilities of the enolization of diketopiperazines and of structures which may be formed by the enolization of polypeptides. Thus, diketopiper-

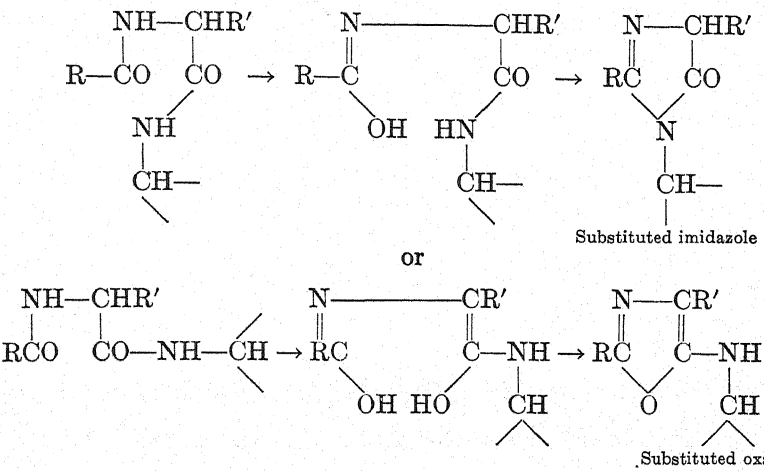
[13] Troensegaard, N., Über die Konstitution der Eiweissverbindungen, *Z. angew. Chem.*, 38: 623–626 (1925); *cf.* also *Z. physiol. Chem.*, 112: 86–103 (1920); 127: 137–185 (1923); 130: 84–86 (1923); 133: 116–125 (1924); 134: 100–112 (1924); 142: 35–70 (1925); 153: 93–110 (1926).

[14] Karrer, P., and Gränacher, C., Über Anhydride von Aminosäuren und Aminosäurederivaten, *Helv. Chim. Acta*, 7: 763–780 (1924); Karrer, P., and Widmer, R., Zur Kenntnis der Aminosäuren, *ibid.*, 8: 203–205 (1925), (*cf.* also 8: 205–211 and 211–217); and Karrer, P., Gränacher, C., and Schlosser, A., Zur Kenntnis der Diketopiperazinderivate, *ibid.*, 6: 1108–1112 (1923).

azine may enolize in two ways, the hydrogen being withdrawn from the imino groups or from attachment to the adjacent carbon.

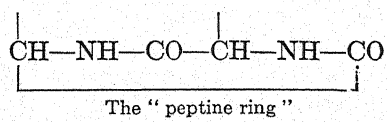


In either case the ring structures which result should have reactions different from those of the diketopiperazines or of normal polypeptides. Similarly, polypeptides may be considered to enolize according to the following scheme:



yielding substituted imidazoles or substituted oxazoles. Aside from the occurrence of the imidazole ring in histidine, the presence of imidazoles or oxazoles in the normal structure of proteins still remains to be proved.

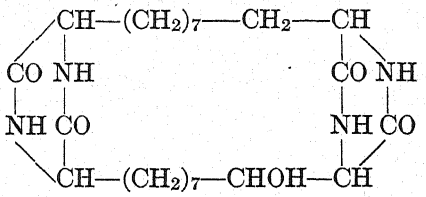
6. *The Peptine Ring Hypothesis.*—Ssadikow and Zelinsky [15] are in general agreement with the other workers who insist that the constitution of the protein molecule is not that of a long polypeptide chain but rather that of a system of cyclic structures. They propose a "polypeptine" unit with its basis as the peptine ring, *i.e.*, a substituted piperazine,



[15] Ssadikow, W. S., and Zelinsky, N. D., Über Produkte der katalytischen Spaltung von Eiweissstoffen, *Biochem. Z.*, 136: 241–249 (1923).

the only difference from the diketopiperazine theory being that they suggest that these rings are held together not by secondary valences but by long hydrocarbon chains and that cyclic or heterocyclic rings may be attached to the side chains.

The essence of their theory is that the linkage between the carboxyl and the amino groups forms cyclic compounds rather than straight-chain compounds and that the ring structures which are formed, in general, may contain more than one diketopiperazine ring, as is indicated in the following structure which they believe represents a compound that they isolated by the partial hydrolysis of proteins.



In a later paper, Ssadikow [16] suggests different types of ring structures as characterizing the behavior of different proteins toward enzymes. His *collagen* type is characterized as being readily digested by pepsin but resistant to the action of trypsin, and he suggests that this protein may be built up of four peptine rings, containing α, β, δ, and γ linkages. The α-linkage is formed between two $-CH_2OH$ groups with the elimination of water, the β-linkage between two $-C(OH)$ groups with the elimination of water, the δ-linkage between a $-CH_2OH$ group and a $-COOH$ group with the elimination of water, and the γ-linkage is an enolized peptide linkage. He suggests that the α-, β-, and δ-linkages are hydrolyzable by trypsin, and though normally the peptide linkage would be hydrolyzed by trypsin, the fact that it is enolized and the enolic group united through a β-linkage makes it trypsin-stable.

His *keratin type* of protein is stable to both pepsin and trypsin. He interprets this to mean that there are no α-, β-, or δ-linkages and that the γ-linkage is blocked, but not through a β union. He accordingly postulates a η- or a υ-linkage through a nitrogen atom, all three valences of which are attached to carbon. His η-linkage is formed by the elimination of water between the —OH of an enolized peptide linkage and the hydrogen of the —NH group of another peptide linkage. His υ-linkage is formed by the elimination of water between an aliphatic hydroxy group and the hydrogen attached to the nitrogen of a peptide linkage.

Although admittedly the entire argument presents a hypothesis,

[16] Ssadikow, W. S., Über einige Bindungsarten im Eiweissmolekül, *Biochem. Z.*, 179: 326–331 (1926).

nevertheless, so far as the author is aware, this is the first attempt to explain the specific reactions of proteins toward trypsin and pepsin in terms of structural organic chemistry, and for that reason the hypothesis is reproduced.

7. *The Oriented Long Polypeptide Chain Theory.*—Dorothy Jordan Lloyd [17] has been an ardent exponent of the theory that collagen, silk, wool, and other protein fibers and those proteins giving an X-ray diffraction pattern possess a structure similar to that shown in Fig. 123. In this figure three polypeptide chains have been laid close together, the middle one running in the reverse direction from the two outer chains. Miss Lloyd points out that the carbon-to-carbon bonds are at an angle of approximately 109° and the valence bonds of nitrogen

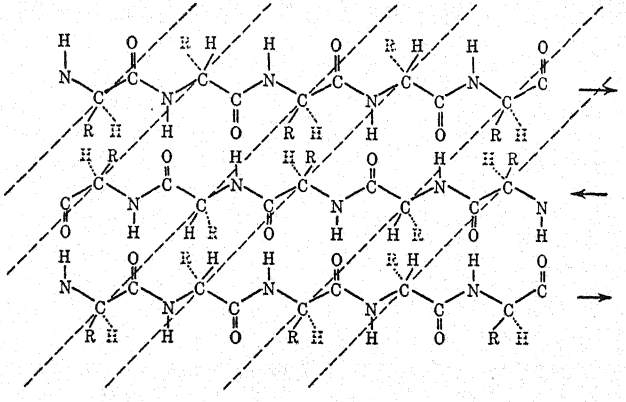


FIG. 123.—A possible space arrangement of polypeptide chains in relation to each other, as postulated for fibrous proteins, tendons, collagen, etc. Note the primary linkages within the peptide chains and coordinate linkages which hold the chains in the biological structure. (After Dorothy Jordan-Lloyd.)

have approximately a 120° angle. Accordingly the atoms are probably oriented around a spiral, and in a plane surface diagram would be represented by a zigzag line more or less approximating that in Fig. 123. We have already seen that the spatial arrangement of the R, NH_2, COOH, and H linkages around the asymmetric carbon of all amino acids is the same. Consequently in the diagram of Fig. 123, alternating "R" and "H" groups will lie first above the plane of the paper and then below the plane of the paper. Therefore, the space arrangements will tend to be repeated in a 1, 3, 5, . . . amino-acid sequence, thus making for a regularly repeated pattern of a three-dimensional solid body with the unit patterns lying in regular planes. This

[17] Lloyd, D. Jordan, Colloidal Structure and Its Biological Significance, *Biol. Rev.*, 7: 254–273 (1932); *cf.* also various papers on the swelling of proteins (*loc. cit.*)

would account for the similarity in the X-ray diffraction patterns which have been observed both with the fibrous and the non-fibrous (globular) proteins irrespective of their source, particle weight, etc. In her earlier papers Miss Lloyd refers to the "cross attractions" which must occur between the electropositive —NH and the electronegative —CO groups. Recently Wrinch and Lloyd [18] and Huggins [19] have independently suggested that the linkage between these groups is in all probability of the hydrogen bond type, and this seems to be exceedingly probable. The dotted lines in Fig. 123 indicate planes composed of regularly repeated atomic groupings. Similarly the "backbone" of the polypeptides contains a series of regularly repeated atomic units, and it is suggested that it is these planes which give rise to the X-ray diffraction patterns.[20]

A structure such as is shown in Fig. 123 accounts admirably for the phenomena of protein hydration [21] and for tensile strength behavior. Fibrous proteins swell greatly in the cross-sectional direction but only elongate slightly in the longitudinal direction. This is explained by water entering in between the "backbone" chains and separating the polypeptide chains from each other, whereas in the longitudinal direction the primary valence bonds prevent any great elongation. Tensile strength decreases greatly across the swollen fiber but is not proportionally decreased in the direction of the fiber length. The X-ray diffraction patterns of the swollen fiber indicate a major displacement of the dimensions across the fiber but relatively little displacement in the direction of fiber length.

It will be further observed from an inspection of Fig. 123 that the arrangement of the amino-acid units simulates a cyclic diketopiperazine and that accordingly the X-ray evidence for a diketopiperazine structure may arise not from true diketopiperazines but from these specially oriented polypeptide chains.

8. *The Globular Protein Hypothesis.*—We have already referred to the ultracentrifugal studies of Svedberg and his measurements of the micellar weight of protein sols. Certain of his micellar weights have been given in Table IX. In his studies on the "molecular weights" of proteins he has in a number of instances measured the shape factor

[18] Wrinch, D. M., and Lloyd, D. J., The Hydrogen Bond and the Structure of Proteins, *Nature*, 138: 758–759 (1936).

[19] Huggins, M. L., Hydrogen Bridges in Organic Compounds, *J. Org. Chem.*, 1: 407–456 (1937).

[20] Clark, G. L., and Schaad, J. A., X-ray Diffraction Studies of Tendon and Intestinal Wall Collagen, *Radiology*, 27: 339–356 (1936); Clark, G. L., and Shenk, J. H., X-ray Diffraction Studies of Globular Proteins, I–III, *ibid.*, 28: 58–67, 144–156, and 357–361 (1937).

[21] Lloyd, D. J., Absorption of Water by Gelatin. VI., *Biochem. J.*, 25: 1580–1592 (1932); The Properties of Collagen Fibres, *J. Soc. Chem. Ind.*, 51: 141–147T (1932).

and concludes that many proteins are "globular" in form. As Sörensen points out, "such long polypeptid chains doubtless will be flexible and apt to roll themselves up by reason of the cohesion forces by which the various parts of the polypeptid chain attract each other." Still more recent evidence [22] indicates that the surface denaturation of egg albumin is brought about when the egg-albumin molecule unrolls in the surface film.

Svedberg's ultracentrifugal studies indicate that there are relatively few "molecular weight" groupings. Svedberg [23] sums up his studies in the following words: "We have found that the molecules of most of the homogeneous native proteins are simple multiples or submultiples of 34,500, which is the molecular weight of ovalbumin. Only a very limited number of different molecular weights are represented among the proteins. On the other hand, we know a large number of proteins differing widely with regard to chemical composition, isoelectric point, and light absorption. This means that chemically different proteins may have the same (or nearly the same) molecular weight. As a matter of fact we find that the numerous proteins fit into a few molecular weight classes. Recent investigations have shown that this regularity probably obtains from the lowest molecular weight so far observed for a protein, or 17,000, up to the highest weight, or 5,000,000. It seems that just about a dozen different steps are required in order to proceed from the lowest to the highest weight. With increasing weight the absolute interval between the steps becomes larger and larger. Thus there are six different molecular weights between 17,000 and 200,000, and also six between 300,000 and 5,000,000. What reality—chemical or physiological—is the explanation of this puzzling display of figures we do not know as yet." Since this was written the virus proteins (*vide infra*) have been investigated, and these appear to have particle weights in the neighborhood of 20,000,000.

The disturbing thing about Svedberg's "molecular weights" is that there is a "stability range" of *p*H over which the proteins are stable and sediment with a uniform velocity, whereas at *p*H's greater or less than this stability range the molecules undergo (in Svedberg's terminology) "decomposition" into smaller fragments which are in most instances one-half, one-quarter, etc., units of the original "molecular weight." This "decomposition" is reversible, however, and when the

[22] Neurath, H., The Influence of Denaturation on the Spreading of Proteins on a Water Surface, *J. Phys. Chem.*, 40: 361–368 (1936); *cf.* also Fosbinder, R. J., and Lessig, A. E., *J. Franklin Inst.*, 215: 579–592 (1933); Gorter, E., and Grendel, F., *Proc. Acad. Sci. Amsterdam*, 29: 371–382 (1926); Gorter, E., and Philippi, G. Th., *Proc. Acad. Sci. Amsterdam*, 37: 788–793 (1934); Gorter, E., van Ormondt, J., and Dom, F. J. P., *Proc. Acad. Sci. Amsterdam*, 35: 838–843 (1932).

[23] Svedberg, The., Sedimentation of Molecules in Centrifugal Fields, *Chem. Rev.*, 14: 1–15 (1934).

"decomposed" protein fragments have been reprecipitated and reworked and brought into the *p*H stability zone, the original sedimentation velocity and the original "molecular weight" are regained.[24]

The author believes that Svedberg is using the term "molecular weight" in the physical sense of a particle which has the mean kinetic energy of a molecule (*vide supra*) and that the "molecular weights" are not in accord with the definition of the chemist, *i.e.*, the smallest unit of a substance which is capable of independent existence. Sörensen (*loc. cit.*) has pointed out that Svedberg's "decomposition" is in harmony with his own theory of reversible dissociable component systems (*vide supra*). It is also in agreement with the theory of protein peptizability (*vide supra*). On this theory Svedberg's "molecular weights" would be dependent on the method of preparation and the ionic environment of the protein micelle. They would be micellar weights. With different methods of preparation and with different ionic environments, peptization (Svedberg's "decomposition") would take place into smaller units, eventually reaching a point where no particles would sediment under the gravitational forces developed in the ultracentrifuge. If polypeptide chains are held together by hydrogen bonds in the protein aggregate, the hydrogen and hydroxyl ions would be the most potent units tending to distort or loosen the hydrogen bonds, and these are the units which Svedberg has found to bring about his protein "decomposition" most readily. An alteration of the hydrogen-ion concentration should reestablish the hydrogen bond linkages between polypeptide chains and in a standard environment again produce micelles of essentially the original size.

As we have already seen, there is an accumulating body of evidence that the protein fractions which we can isolate under laboratory conditions are not preformed as such in the original biological material but are artifacts produced by laboratory manipulation,[25] and this must be taken into consideration in the interpretation of the challenging series of data which Svedberg has presented in his numerous papers.

One additional point deserves further consideration before Svedberg's data can be accurately interpreted. His stability zone lies in the region close to the isoelectric point of the proteins, and his "decomposition" range lies in regions where the proteins will be ionized and accordingly possess excesses of either positive or negative charges. In the light of Levine's (*loc. cit.*) calculations the "decomposition" may not be real but may result from the repulsion of like-

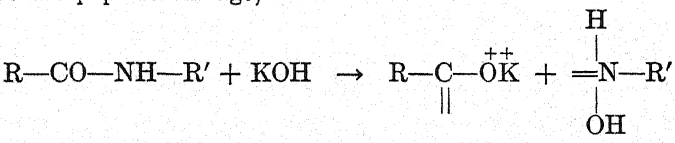
[24] Among numerous papers of Svedberg where this phenomenon has been noted, see, for example, *J. Am. Chem. Soc.*, 50: 3318 (1928).

[25] Block, R. J., *J. Biol. Chem.*, 105: 455 (1934), says, "Proteins as prepared in the laboratory are artificial products produced by the reagents employed in their preparation."

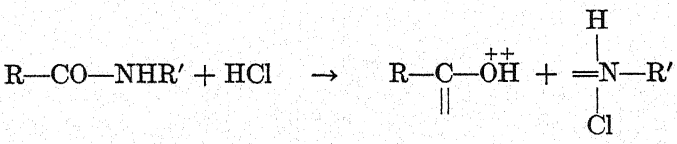
charged particles which prevent sedimentation in the gravity field. Furthermore, when calculating sedimentation rates it may not be justifiable to ignore the electrical forces between particles at or close to the isoelectric point, for proteins are not electrically neutral at the isoelectric point, but in reality this is the point of their maximum ionization. Here they exist as zwitterions, the negative charges balancing the positive charges within the molecule. Whether or not the zwitterion constitution will have a profound bearing on sedimentation velocity remains to be determined. Certainly it would have a profound bearing on forces which favor or inhibit aggregate formation. We shall refer to some of these problems again when we discuss the "virus proteins."

9. *Sörensen's Reversible Dissociable Component Systems.*—We have discussed this theory in the preceding chapter, inasmuch as it has a profound bearing on problems of protein classification, and the student is referred to that discussion which is likewise pertinent to the present consideration of the theories of protein structure.

10. *The "Akropeptide" Theory.*—Robertson,[26] on the basis of a series of studies dealing with the electrical conductivity of proteins in solutions of acid and alkalies, the electrical migration of proteins, the equivalent conductivities of protein solutions, etc., reached the conclusion that proteins dissociate into ions, the dissociation taking place at the peptide linkage,



and



Thus, on the addition of one equivalent of KOH (or HCl, the same argument applies to acidic as to basic solutions), the protein dissociates at the middle peptide linkage into two massive protein ions of essentially the same size, one being charged positively, the other negatively. The addition of a second equivalent of alkali would dissociate each of these ions approximately at the middle peptide linkage again into two ions, so that the addition of 1, 2, 3, 4, . . . equivalents of alkali would give rise respectively to 2, 4, 8, 16, . . . protein ions. Robertson's theory was dependent on a tautomeric keto $\rightleftharpoons$ enol

[26] Robertson, T. Brailsford, The Physical Chemistry of the Proteins (483 pp.), Longmans, Green and Company, New York (1918).

shift at the peptide linkage but went further than this in that it postulated actual ionization at the linkage. He presented a great deal of evidence in support of his theory and the theory had a certain vogue at the time it was promulgated, but it early fell into the discard because of the improbability of the peptide linkage opening up in the manner that he postulated.

Recently Fodor, as the result of a series of investigations on the structure of proteins, has proposed an akropeptide theory which has a remote resemblance to Robertson's theory. Fodor [27] investigated the constitution of a number of proteins, chiefly gelatin and casein, by heating them with acetic anhydride or with glycerol or resorcinol at 130° to 150° for 2 or 3 hours. Under these conditions he reports that the proteins are broken down into large polypeptide units. He reports the isolation of units of a tetrapeptide, of a ditetrapeptide, and of a tetratetrapeptide and believes that the ditetrapeptide is formed by the combination of two tetrapeptides and that the tetratetrapeptide is formed by the combination of two ditetrapeptides. In the place of enolization at the peptide linkage, as Robertson postulated, Fodor assumes an enolization of the following type,

$$\mathrm{R{-}CO{-}NH{-}R' \rightarrow R{-}\underset{|}{C}(OH){-}\underset{|}{N}{-}R'}$$

whereby two free valence bonds are liberated. These free valences, instead of forming a double bond linking carbon to nitrogen, bridge across with similar valences in another carbon chain, giving the following structure:

$$\begin{array}{l} \mathrm{R{-}\underset{|}{C}(OH){-}\underset{|}{N}{-}R'} \\ \mathrm{R'{-}N{-}(OH)\underset{|}{C}{-}R} \end{array}$$

which is the characteristic linkage in his akropeptides. Thus, two dipeptides each of which has one enolized peptide linkage could unite to form a tetrapeptide. The enolization of one peptide linkage in this tetrapeptide could unite with a similar monoenolized molecule to form a ditetrapeptide. Again one equivalent of an enolizing reagent would cause the ditetrapeptide to give rise to a tetratetrapeptide, so that on the addition of 1, 2, 3 equivalents of an enolizing reagent there would arise polypeptides containing 4, 8, and 16 amino-acid residues. Fodor notes that, since this akropeptide linkage is formed

[27] For a summary see Fodor, A., and Kuk, S., Über den Zusammenhang zwischen dem chemisch-strukturellen und dem kolloiden Bau der Proteine, *Kolloid Z.*, 74: 66–87 (1936); *cf.* also Fodor, A., *et al.*, "Über die chemische Struktur einiger Proteine, I–XII, *Z. physiol. Chem.*, 171: 222–241 (1927); *Biochem. Z.*, 200: 211–222, 223–235 (1928), 210: 24–41 (1929), 214: 242–252 (1929), 222: 226–239 (1930), 228: 310–314, 315–326 (1930), 240: 123–139, 140–148 (1931), 245: 350–361 (1932), 259: 331–339 (1933).

by the process of enolization of the peptide linkage, it is a labile linkage and may, with one set of reagents, give rise to an increasing complexity in the molecular size of the polymerization product, and with another set of reagents high-molecular polymers may be broken down, giving rise to simpler products, and he believes that glycerol and resorcinol have acted as depolymerizing reagents.

He reports the isolation and characterization of akropeptides of which the following is typical: (leucylprolyllysylglutamyl)-(leucylprolylalanylglutamyl), $C_{41}H_{67}N_9O_{12} \cdot 2H_2O$, where the quantities in

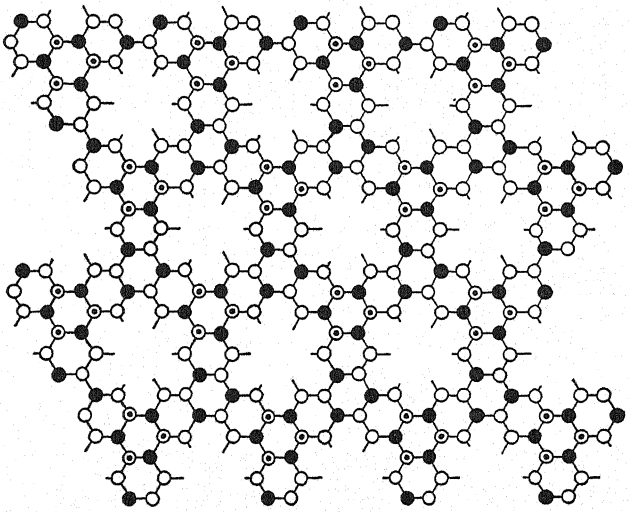


FIG. 124.—The Wrinch "cyclol pattern" for proteins. The median plane of the lamina is the plane of the paper; the lamina has its "front" surface above and its "back" surface below the paper.

parentheses represent the tetrapeptides united in the ditetrapeptide.

11. *The Cyclol Theory.*—The latest theory of protein structure is that proposed by Miss Wrinch [28] based on a "cyclol" pattern which involves essentially a series "of diazine and triazine hexagons, built into one another in such a way that every triazine hexagon is sur-

[28] Wrinch, D. M., On the Pattern of Proteins, *Proc. Roy. Soc.* (*London*), 160: A, 59–86 (1937); On the Molecular Structure of Chromosomes, *Protoplasma*, 25: 550–569 (1936); On the Structure of Insulin, *Science*, 85: 566–567 (1937); *cf.* also Langmuir, I., Schaefer, V. J., and Wrinch, D. M., Built-up Films of Proteins and their Properties, *Science*, 85: 76–80 (1937).

rounded by three of the diazine type and pairs of triazine hexagons are joined by a single diazine hexagon." Figure 124 shows Miss Wrinch's cyclol pattern. She observes that this pattern can be derived from the open polypeptide chain by a cyclization involving the enolization of the peptide linkage and that the cyclols fall into a progressive series containing 6, 18, 30, 42, 54, 66, 78, 90, 102, . . . $(6 + 12n)$ amino-acid residues. Figure 125 shows the simplest "cyclol-6" molecule and Fig. 126 shows "cyclol 42" arrangement which has resulted from the polymerization of seven "cyclol-6" molecules.

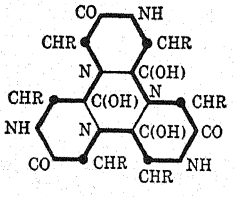


FIG. 125.—A "6-cyclol." (After Wrinch.)

Miss Wrinch developed her theory largely from a consideration of mathematical probabilities, X-ray crystal structure data, geometrical isomerism, protein crystal symmetry, and the structure and behavior of unimolecular protein films as measured and studied by means of the Langmuir surface tension balance. She points out that the cyclol theory accounts for most of the physicochemical properties of proteins which have been investigated, including the breaking up of the Svedberg "molecules" into submultiple "molecular" units. The arrangement shown in Fig. 124 is regarded as a sheet of amino-acid

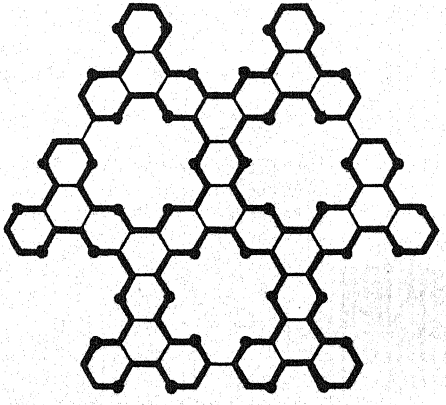


FIG. 126.—A "42-cyclol." (After Wrinch.)

aggregates, polymolecular in length and breadth but monomolecular in depth. In the cyclol pattern a part of the hydroxyl groups are directed downward from the cyclol plane and a part are directed upward above the plane. Linkages between the cyclol sheets are accordingly formed through the hydrogen bond or other similar forces linking electropositive groups across to electronegative groups. Thus, accord-

ing to the cyclol theory, proteins would be built up of a series of lamina, and these lamina may be linked together by means of intermolecular attractions, possibly in the same way that the linkage of structural planes within clay minerals are linked. Proteins therefore can swell largely in the cross-sectional direction, but in the plane of the cyclol sheet the degree of swelling will be determined by the tensile strength of the cyclol bonds and the amount of distortion which the cyclol structure can undergo under the tensile forces which are impressed upon it.

It is evident that, if the cyclol theory is accepted, it will explain the peptization behavior of the proteins or Sörensen's reversible dissociable component system theory and would likewise explain the possibility of isolating from biological material by a standard technic protein "fragments" which behave as though they were chemical entities, since peptization, dispersion, solubility, etc., would all be simply the separation of cyclol sheets from cyclol sheets.

12. *Bergmann's Theory of Recurring Amino-acid Units.*—Bergmann,[29] from a study of the analytical data of the amino-acid content of cattle hemoglobin, cattle fibrin, egg albumin, silk fibroin, and gelatin, has formulated the hypothesis that all simple natural proteins are constructed on similar structural principles, *i.e.*, "Every individual amino acid residue in the peptide chain of the protein molecule recurs at constant intervals . . . in a characteristic and periodic manner throughout the entire polypeptide chain. . . . The peptide structure is common to all proteins, but the various natural proteins differ from each other in that their different amino acid constituents are represented by different frequencies. Thus, the physico-chemical and the biological properties of a particular protein are based, in the last analysis, on the frequencies with which its constituent amino acid residues recur within the peptide chain."

The reasoning which Bergmann employs may be illustrated from his study of fibrin:

1. Arginine and glutamic acid are present in fibrin in the ratio of 32 : 72.

2. This ratio may be expressed (inverted) as 9 : 4, 18 : 8, 36 : 16, 72 : 32, etc.

3. Therefore, considering only glutamic acid, there may be 4, 8,

[29] Bergmann, M., Complex Salts of Amino Acids and Peptides. II. Determination of *l*-Proline with the Aid of Rhodanilic Acid. The Structure of Gelatin, *J. Biol. Chem.*, 110: 471–479 (1935); Bergmann, M., and Niemann, C., On Blood Fibrin. A Contribution to the Problem of Protein Structure, *ibid.*, 115: 77–85 (1936); On the Structure of Proteins: Cattle Hemoglobin, Egg Albumin, Cattle Fibrin, and Gelatin, *ibid.*, 118: 301–314 (1937); and Bergmann, M., and Fraenkel-Conrat, H., The Role of Specificity in the Enzymatic Synthesis of Proteins. Syntheses with Intracellular Enzymes, *ibid.*, 119: 707–720 (1937).

16, 32, etc., individual units of glutamic acid present in the protein molecule.

4. The question as to which is the correct value Bergmann ascertains by the expression

$$\frac{F \times AW}{MW} = \frac{100}{\%}$$

where F = the " frequency " of the particular amino acid being studied;
AW = the *average* molecular weight of all the amino acids resulting from a protein hydrolysis;
MW = the molecular weight of the particular amino acid whose " frequency " is being determined,
$\%$ = the percentage of the particular amino acid in the hydrolysate.

5. In the instance of glutamic acid which is being considered, "frequencies" of 4, 8, 16 yield "average molecular weights" of 260, 130, 65, respectively. The first value is too high, the last value too low to be possible. Therefore, *glutamic acid forms one-eighth of the amino acid residues in fibrin.*

6. It is obvious that when this line of reasoning is extended to *all* of the amino acids we will arrive at a certain *minimum* number of residues for each amino acid *all of which must, of course, be expressed as whole numbers*, for there can be no partial molecules of an amino acid in the polypeptide chain.

7. The actual *molecular weight* of the protein must be either the molecular weight of the polypeptide containing the *minimum* number of individual amino acid residues as ascertained in item 6 or must be some simple whole-number multiple of this minimum size.

8. Bergmann then goes further and *postulates* that the "frequencies" represent also "position numbers" and that the amino acids recur at *constant intervals* throughout the entire polypeptide chain, these constant intervals being expressed by their "frequencies" as determined by the F in the expression in item 4. Thus, in the case of glutamic acid already cited, it will recur in the fibrin molecule as every eighth amino acid residue.

In the case of fibrin Bergmann finds the following frequencies: glutamic acid 8, lysine 12, arginine 18, aspartic acid 18, proline 18, tryptophane 32, histidine 48, methionine 48, and cystine 64, with a *calculated* minimum polypeptide chain length of 576 amino-acid residues which would have a molecular weight of 69,300. In the case of egg albumin the frequencies found were: glutamic acid 8, aspartic acid 18, methionine 24, lysine 24, arginine 24, tyrosine 36, histidine 72, and cysteine 72. These data yield a "calculated" molecule of 288 amino-acid residues and a "minimum molecular weight" of 35,700,

which is in excellent agreement with Sörensen's 34,000 and with Svedberg's 34,500.

Therefore, according to Bergmann, the naturally occurring proteins appear to be built up of $n \times 288$ amino-acid residues. He suggests that the simple proteins, egg albumin, insulin, Bence-Jones protein, pepsin, and trypsin, which show a Svedberg molecular weight of 34,500, in reality contain this basic unit of 288 amino acid residues, and the true molecular weight would be 288 $\times$ (the average molecular weight of the amino acid residue). Therefore proteins with low (or with high) average molecular weight residues will deviate considerably from the Svedberg unit weight.

The next higher series appears to be those proteins containing 576 amino-acid residues; others contain 1,152 residues, etc., and accordingly would (approximately) give rise to the $2 \times 35{,}000$ and $4 \times 35{,}000$ molecular weights characteristic of Svedberg's determinations.

The recurrence of amino acids in the chain at regularly spaced intervals may be illustrated in respect to silk fibroin. To quote Bergmann,[30] "Each glycine residue in silk fibroin is separated from the adjacent glycine residues by an amino-acid residue other than glycine, *e.g.*,

-G-X-G-X-G-X-G-X-G-X-G-X-G-X-G-X-

Each alanine residue is separated from the adjacent alanine residues by three other residues, *e.g.*,

-A-X-X-X-A-X-X-X-A-X-X-X-A-X-X-X-A-X-X-X-A-

Each tyrosine residue is separated from the adjacent tyrosine residues by fifteen other residues, *e.g.*,

-X-T-X-X-X-X-X-X-X-X-X-X-X-X-X-X-X-T-X-X-X-X-X-X-X-X-X-X-X-X-X-X-T-

On combining the above configurations, the structure of a segment of the silk fibroin molecule is obtained, *i.e.*,

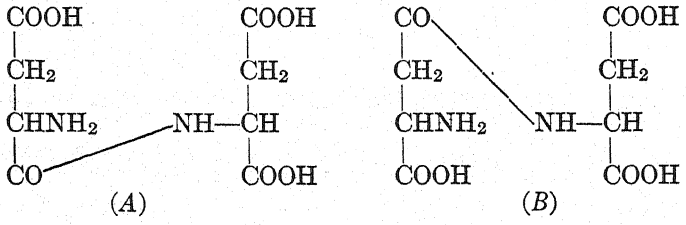
-G-A-G-X-G-A-G-X-G-A-G-T-G-A-G-X-G-A-G-X-G-A-G-X-G-A-G-T-G-"

Bergmann admits that the regular spacing of amino acid units at definite intervals is only a theory but notes that it has some support in the case of gelatin from which prolylglycine-containing polypeptides have been isolated in considerable amounts and where the space-relationship theory demands —P—G— juxtapositions. In any event, these new theories of Bergmann are challenging and will unquestionably stimulate much thought and research.

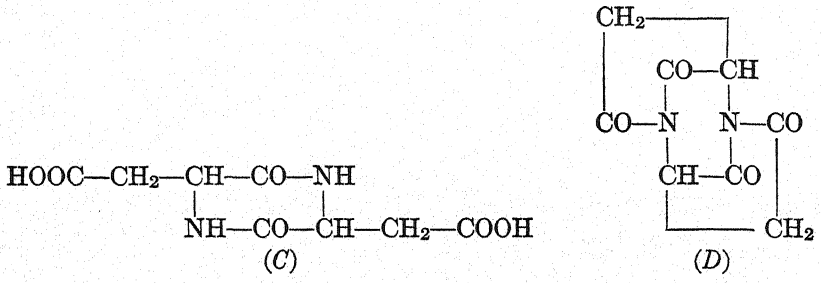
PEPTIDES OF THE DICARBOXYLIC ACIDS.—In addition to the above theories, there is a further transformation involving ring structures, which appears to be characteristic of the dipeptides of aspartic and

[30] Bergmann, M., and Niemann, C., Newer Biological Aspects of Protein Chemistry, *Science*, 86: 187–190 (1937).

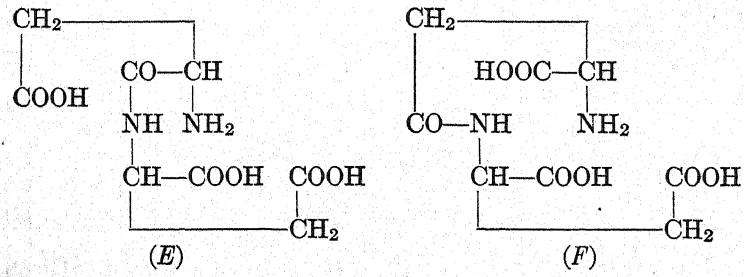
glutamic acids. Blanchetière,[31] in studying the behavior of dipeptides of aspartic and glutamic acids, isolated two products, one of which appears to be the normal dipeptide in which the peptide linkage is through the carboxyl and amino groups attached to the α-carbon atoms, as shown in (*A*). The other or ω-peptide is characterized by the peptide linkage being formed by the union of the α-amino with a β-carboxyl group, as shown in (*B*).



When these two forms were converted into anhydrides, one formed a normal substituted diketopiperazine, as shown in (*C*), the other a system of three condensed rings, as shown in (*D*).



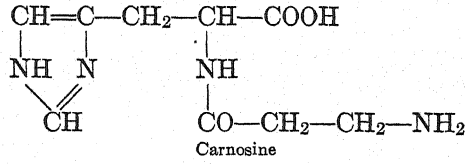
Compound (*D*) undergoes hydrolysis, yielding two types of dipeptides (*E*) and (*F*).



Engeland isolated, in 1908, a β-amino acid, β-alanine, from muscle "press-saft." Blanchetière points out that while β-amino acids have

[31] Blanchetière, M. A., Constitution des anhydrides des acides aspartique et glutamique. Son importance biologique, *Bull. soc. chim. biol.*, 6: 854–860 (1924).

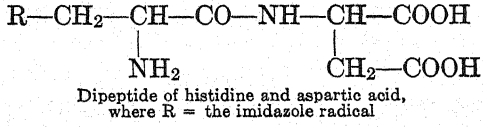
not been found in proteins, they do occur in other compounds characteristic of living material, such as carnosine (β-alanylhistidine).



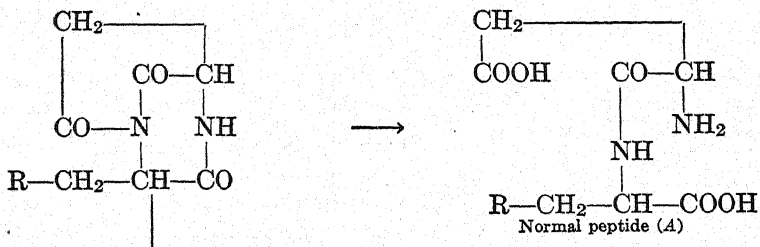
Carnosine

The mechanism which he proposes is an initial dipeptide between histidine and aspartic acid, which in turn condenses to an internal anhydride capable of hydrolyzing to yield two different compounds, peptide (A) and peptide (B). Peptide (A) is the normal peptide which one would expect between histidine and aspartic acid. Peptide (B) is abnormal. He notes that peptide (B) is very sensitive to reagents and readily undergoes decarboxylation, losing carbon dioxide at (x), to yield carnosine.

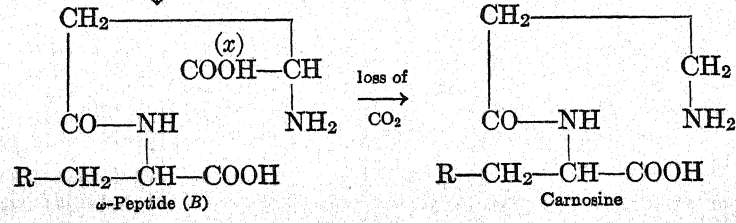
Blanchetière points out that apparently all the ω-peptides are characterized by the great ease with which they lose carbon dioxide. The fact that Blanchetière has prepared the double anhydrides containing three condensed rings is at least suggestive that such compounds may occur more generally in nature than we have believed.



Dipeptide of histidine and aspartic acid, where R = the imidazole radical



Normal peptide (A)



ω-Peptide (B)

Carnosine

THE POSSIBILITIES OF PROTEIN ISOMERISM.—We have already noted in a consideration of the polypeptides that there are six possible isomers of the tripeptide containing tyrosine, leucine, and alanine, and that, in order to identify a naturally occurring tripeptide containing these amino acids, it would be necessary to synthesize the various isomers to determine the space configuration of the polypeptide which had been isolated.

The number of possible isomers of a tripeptide is accordingly $\underline{|3}$. For a tetrapeptide we would have $\underline{|4}$, or 24 possible isomers.

Assuming a protein to be made up of 20 amino acids, and *assuming that each amino acid occurred only once in the protein chain,* there would still remain the possibility of having $\underline{|20}$ or approximately 24×10^{17} different compounds, each containing the same amino acids in identical proportions and differing only in space relationships.

According to modern viewpoint, proteins have a very much greater molecular weight than would be found for such a "polypeptide." Egg albumin, according to the best evidence, appears to have a molecular weight of about 34,000, so that presumably an egg-albumin molecule is made up of several hundred molecules of individual amino acids. Regardless of how these are linked together, it must be obvious from the above that there are limitless theoretical possibilities to the number of compounds which could be synthesized from the known list of amino acids.

Leathes [32] calls attention to these possibilities in a very striking way. He states, "Supposing it were a chain of only 50 links, a very simple case; if all the links were different the number of possible permutations is denoted by the innocent-looking symbol $\underline{|50}$. If, instead of all being different, one kind of link recurred ten times, the number would be reduced to $\underline{|50}/\underline{|10}$. If, in addition, there were 4 that recurred four times and 10 that recurred twice, it would be further reduced to

$$\underline{|50} / \underline{|10} \times (\underline{|4})^4 \times (\underline{|2})^{10}$$

It would now consist of a chain of only 50 links, of which there were only 19 different kinds, and the number of different arrangements of its parts would be about 10^{48}. Astronomy deals with big figures. Light, it is said, takes 300,000 years to travel from one end of the Milky Way to the other; this distance expressed in Ångström units, 10,000,000 of which go to a millimeter, would be less than 10.[32] So far are we from knowing the structure of protein molecules. So far are we from knowing what variations in disposition of the parts

[32] Leathes, J. B., Function and Design, *Science,* 64: 387–394 (1926). (Every biochemist or biologist interested in vital phenomena should read this paper.)

in such a molecule may not occur without our being within a measurable distance of detecting them. For if the number of possible varieties of a protein whose molecular weight is known, and known to be exceptionally small, and which contains the several amino acids in a known proportion, is as great as this, the number that is possible when that proportion may be changed is practically incalculable, each change in proportion being capable of a number of new arrangements that could be calculated, as was done for our hypothetical case. . . . The peculiar thing about the chemistry of living matter is not that the reactions that are characteristic in it are novel, but that in the rough and tumble of ordinary liquid systems their occurrence is almost infinitely improbable. Where there is life circumstances exist which make them the rule. . . . Forces which determine the relative positions of adjacent foreign molecules and so affect their behavior are something to which there is no analogy in the growth of crystals in a saturated solution."

Leathes' calculations do not take into consideration the possibility of various linkages between the various amino acids, keto $\rightleftharpoons$ enol isomerism, cyclic structures, such as have been discussed in the preceding part of this chapter, etc., all of which would introduce further possibilities in calculating the number of possible isomers which could be formed from a given number of amino acids.

Miss Wrinch discusses the molecular structure of chromosomes. The genes, which are regarded as the genetic units, are located in the chromosomes. She considers a pattern of protamine combined with nucleic acid, and (assuming that there are only six types of constituents and that five-sevenths of the constituents belong to one type as is the case with the protamine, clupeine) she notes that, if the protein pattern extended for a distance of 40 μ, the total number of possible patterns would approximate the inconceivable figure of $10^{50,000}$. Beside a figure of this magnitude even astronomical distances fade into insignificance.

As we shall see later, the biological reactions of the proteins enable us to differentiate rather sharply, in most instances, between the proteins which are characteristic of one species and those which are foreign to that species, and it is upon these observations that the entire modern structure of immunology has been built. Some workers point out that the species specificity which is demanded by the modern theories of immunology, and which is shown in immunological reactions, is almost infinite and therefore surprising. The author believes with Leathes that, considering the infinite possibilities of the linkages of amino acids in proteins, it is much more surprising that nature should ever synthesize twice in succession proteins which are even remotely alike.

Perhaps no more striking illustration of the exactness with which

vital reactions are regulated in the living protoplasm can be given than the fact that, through untold generations, a given organism reproduces the same structural configurations in the proteins which characterize that organism. The fact that each species has a different set of proteins is not the surprising thing. The surprising thing is that nature is able to control the synthesis of proteins within a single species, so that the same proteins are synthesized by all members of the species.

CHAPTER XVII

CHARACTERISTIC PROPERTIES OF PROTEIN SYSTEMS

As already indicated, the reactions of protein systems must be not only those which are characteristic of the organic groups making up the protein molecule but also those which would be expected of lyophilic colloid systems. The literature in this field is so voluminous that the properties of only a few proteins can be mentioned and these very briefly and inadequately. It is hoped, however, that it will be possible to consider in a casual way some of the more important phenomena which are involved.

I. The Albumins.—Egg albumin and serum albumin are the two proteins of this class which have been most extensively investigated. Both egg albumin and serum albumin can be obtained in crystalline form. Accordingly, they are usually regarded as chemical entities. Although the fact that the albumins could be obtained in crystalline form has been known for many years, the first precise study of the crystallization process was made by Sörensen[1] in 1917.

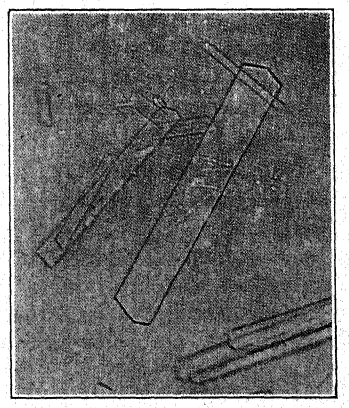


Fig. 127.—Crystals of egg albumin. (After Sörensen.)

Sörensen's technic involves the addition of one volume of neutral saturated ammonium sulfate solution to one volume of clear egg white. The mixed solution is filtered and saturated ammonium sulfate solution added to the filtrate to the point of incipient turbidity. To this solution there is then added 0.2 *N* sulfuric acid solution very slowly, with rapid stirring. The initial turbidity disappears, then a precipitate forms which dissolves on stirring, but the solution of the precipitate becomes more and more difficult with continued addition of sulfuric acid until finally a point is reached where all the precipitate is dissolved, but no more would dissolve if additional sulfuric acid were added. At this stage, crystallization will

[1] Sörensen, S. P. L., Studies on the Proteins, I–V, *Compt. rend. trav. lab. Carlsberg*, Vol. XII, 372 pp. (1917); VI, *ibid.*, Vol. XV, No. 2 (1923).

begin within an hour or two and proceed for several days. The crystals may then be filtered by suction and washed with an ammonium sulfate solution, the concentration of ammonium sulfate being adjusted to the point where it will just fail to precipitate protein from the mother liquor. The crystals are then redissolved and recrystallized by repeating the above process. Sörensen found that three recrystallizations were sufficient to free the egg-albumin crystals from all the other proteins characteristic of egg white. The initial crystallization was best carried out at a final *p*H of 4.604, the second recrystallization at a *p*H of 4.652, and the third recrystallization at a *p*H of 4.699. Figure 127 shows the form of the crystals. These crystals contained water of crystallization equivalent to 0.22 gram water per gram water-free egg albumin. Only at a hydrogen-ion concentration of 13×10^{-6} or a *p*H of 4.886 did the crystals contain no surplus of either ammonia or sulfuric acid. At a higher hydrogen-ion concentration, an excess of sulfuric acid was present, and at a lower hydrogen-ion concentration, an excess of ammonia. Figure 128 shows the form of the curves at varying hydrogen-ion concentrations.

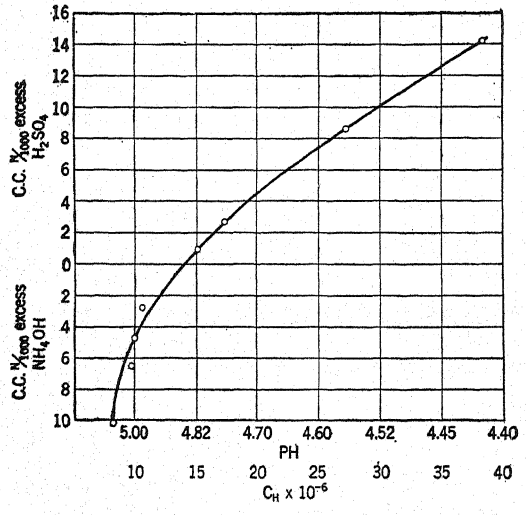


FIG. 128.—Showing the excess acid (H_2SO_4) or alkali (NH_4OH) per milligram equivalents of protein nitrogen present in crystalline egg albumin in equilibrium with solutions having different hydrogen-ion concentrations. (Data of Sörensen.)

The crystals do not contain ammonium sulfate as an integral component. The best-defined crystals, however, are obtained at a hydrogen-ion concentration where a certain amount of sulfuric acid is present in the crystal, so that Sörensen regards the crystal as that of a hydrous egg-albumin sulfate containing 0.22 gram water per gram of dry albumin and approximately 1 equivalent of sulfuric acid per 125 equivalents of protein nitrogen. Such crystals separate at a *p*H of 4.58, which appears to be the optimal *p*H for crystal formation.

In his study of the egg-albumin-ammonium-sulfate-water systems, Sörensen applied the phase rule of Gibbs to the crystallization process.

He points out that other workers have attacked this problem but that in each instance they were working with egg albumin purified by precipitation and not by repeated crystallization. Sörensen finds the Gibbs' phase rule to hold rigidly in an egg albumin-salt-water system, provided that the hydrogen ion is considered as one of the components, and he points out that previous workers failed to secure agreement with the phase rule because they regarded the system as a 3-component system, whereas in reality it is a 4-component system in which equilibrium is reached only at a constant temperature, a constant concentration of ammonium sulfate, and a constant hydrogen-ion concentration. When these three factors are held constant, the content of egg albumin in the mother liquor which is in equilibrium with the crystals will always be the same within experimental error. The state of equilibrium is therefore independent of the initial concentration of protein in the solution, so that from a concentrated albumin solution abundant crystals will form, while from a dilute solution only a small amount of crystals may be expected.

Inasmuch as he was working with a material having a very low diffusion coefficient, equilibrium was very slowly reached, more than 13 days being required in some experiments. Earlier workers had not waited sufficiently long for equilibrium to be attained.

Sörensen investigated certain other factors influencing crystallization and found, as might be expected, that the crystallization begins sooner and proceeds more rapidly as the concentration of ammonium sulfate is increased. When minimal amounts of ammonium sulfate were present, equilibrium was not reached even after 13 days. The final equilibrium in all cases, however, was the same, irrespective of the concentration of ammonium sulfate, *i.e.*, the crystals in each instance had the same chemical composition.

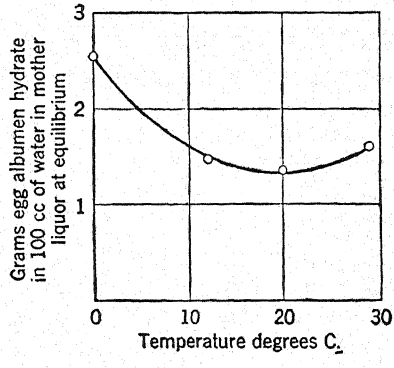


FIG. 129.—The effect of temperature on the crystallization equilibrium of egg-albumin solutions. (Data of Sörensen.)

In a study of the effect of temperature on the equilibrium and on the velocity of crystallization, he found that the velocity of crystallization at 0° was lower than that at 12° or 24°, and that the equilibrium at 0° was essentially different from that at 12° or 24°. Between 12° and 29°, probably at approximately 20°, there is an optimum temperature at which the smallest amount of egg-albumin hydrate is

present in the solution which is in a state of equilibrium with the crystals. Thus, a solution in equilibrium at 0° will produce additional crystals if raised to 29°, while a system which is in equilibrium at 29° will be displaced in the other direction by a transference to 0°, and a part of the crystals will dissolve. Sörensen's equilibrium-crystallization curve at various temperatures is shown in Fig. 129. The solubility at 0° is nearly 100 per cent greater than at 20°.

Within the range of *p*H 4.2 to 5.0 there exists an optimal *p*H concentration at which crystallization, other conditions being equal, will advance the farthest. The optimum point appears to be approximately *p*H 4.58. A decrease in hydrogen-ion concentration from the optimum only causes much less albumin to crystallize. The crystals which do form are normal in shape and character. An increase in hydrogen-ion concentration causes only a slight increase in the solubility of the egg albumin hydrate crystals but does cause a decided alteration in both form and character of the crystals. The crystals contain much more sulfuric acid than normal. At a *p*H 5.22 to 5.30 the crystal form changes from small needle-shaped crystals to prisms. His crystallization curves in relation to *p*H are shown in Fig. 130.

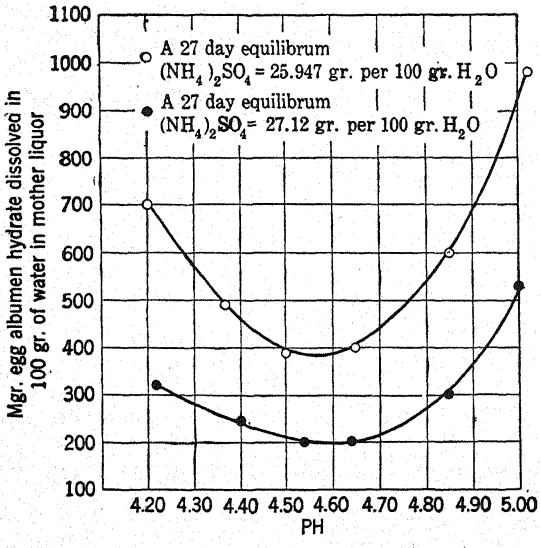


FIG. 130.—Showing the effect of hydrogen ion concentration on the "solubility" of crystalline egg albumin in ammonium sulfate solutions. (Data of Sörensen.)

The forms of the curves in Fig. 130 for the two different concentrations of ammonium sulfate indicate that it should be possible to find a higher concentration of ammonium sulfate at which an increase in *p*H would not affect the solubility of the crystals.

Sörensen's fifth paper is a study of the osmotic pressure of egg albumin measured by the direct method. He finds that an egg-albumin solution of given composition will invariably show one and the same osmotic pressure, and concludes that egg albumin has a molecular weight of about 34,000 and that it exists in solution in the form

of simple molecules. Later, in his paper on reversible dissociable component systems, he concludes that egg albumin also must be regarded as a reversibly dissociable component system but that the dissociation tendency in this system is very slight.

The molecular weight of approximately 34,000 for egg albumin has been confirmed by a number of independent investigations. Svedberg, in the ultracentrifuge, finds it to be 34,500, and measurements of diffusion velocity indicate values of a similar magnitude. Egg albumin, insulin, the Bence-Jones protein, and a few other proteins all appear to have particle weights of approximately 34,000. With the exception of the protamines, these are the lowest particle weights reported for protein preparations, and accordingly 34,000 may be taken as the minimal value which one may anticipate finding for a protein particle weight.

Egg albumin dialyzes slowly through a fairly porous collodion membrane. Lactalbumin dialyzes through a collodion membrane much more readily than egg albumin does. Svedberg in his studies of lactalbumin concludes that it is a heterogeneous protein made up of particles of relatively small molecular weight. No study similar to Sörensen's study of egg albumin has been carried out with lactalbumin. The fact that an egg-albumin solution is monomolecular does not necessitate the abandoning of the viewpoint that egg-albumin solutions show colloidal properties. We have already pointed out that the colloidal realm is defined on the basis of the size of the micelle, irrespective of whether the micelle represents a single molecule or an aggregate of molecules. If the molecule of egg-albumin hydrate is sufficiently large to form a particle having a diameter greater than 1 mμ, then that particle must possess surface forces which are characteristic of the lyophilic colloids. Ultrafiltration experiments indicate very clearly that the egg-albumin molecule lies well within the boundaries of the colloid realm. Consequently, although Sörensen has shown that egg-albumin solutions are molecularly dispersed solutions and that they obey the physicochemical laws, such as the phase rule, nevertheless we cannot abandon the colloid viewpoint in dealing with even egg-albumin systems.

Schutz and Zsigmondy [2] utilized the gold number to characterize the various proteins of egg white. The crude egg white had a gold number of 0.1 to 0.2, crystalline egg albumin 2.0 to 8.0, ovoglobulin 0.02 to 0.05, ovomucoid 0.04 to 0.08, uncrystallized "conalbumin" 0.03 to 0.06. They note that the presence of even a small quantity of the other egg proteins can be detected in the crystalline egg albumin by making use of the gold-number technic.

[2] Schutz, Fr. N., and Zsigmondy, R., Die Goldzahl und ihre Verwertbarkeit zur Charakterisierung von Eiweissstoffen, *Beitr. chem. Physiol. Path.*, 3: 137–160 (1902).

As already noted, egg albumin contains a carbohydrate radical. Numerous investigators have studied the nature of this carbohydrate group. In 1927, Fränkel and Jellinek [3] isolated from egg albumin a disaccharide composed of glucosamine and mannose, in which the union joining the two sugar radicals is through the amino group, inasmuch as the glucosamine-mannose compound does not react to yield nitrogen in the Van Slyke apparatus. Incidentally, they point out that this is the first instance in which mannose has been found in a material of animal origin. Later Rimington,[4] in investigating the carbohydrate complex of the blood serum proteins, isolated a trisaccharide, glucosamine-dimannose. This trisaccharide was non-reducing prior to acid hydrolysis. Serum globulin contained 3.7 per cent of the trisaccharide. Rimington's compound was isolated by the alkaline hydrolysis of the protein. The surprising fact about his trisaccharide is that it was optically inactive. Levene and Mori [5] investigated the carbohydrate in egg albumin and ovomucoid and found it to be a trisaccharide containing one glucosamine residue and two mannose residues. Ovomucoid contained 5.1 per cent, ovalbumin 0.26 per cent. They suggest that the carbohydrate in the three-times-recrystallized ovalbumin may represent contamination with ovomucoid. In a second paper, Levene and Rothen [6] from measurements of the coefficient of diffusion conclude that the carbohydrate molecule is composed of four trisaccharide units and has a molecular weight of approximately 500.

II. The Globulins.—Proteins which have been designated as globulins have been prepared from a great variety of vegetable sources, as well as from muscle tissue, blood serum, and other biological fluids. As has already been indicated, these proteins have been characterized as being soluble in dilute salt solutions and insoluble in pure water. The general method of preparation is to extract the tissue with approximately 10 per cent sodium chloride solution and to dialyze the extract. The proteins which are precipitated will be regarded as globulins, whereas the albumins remain in solution. A modification of this method is to add to the extract an equal volume of saturated ammonium sulfate solution, the globulins precipitating in half-saturated ammonium sulfate, the albumins remaining in solution. The precipitated protein is then redissolved and reprecipitated by half

[3] Fränkel, S., and Jellinek, C., Über die sogenannte Kohlehydratgruppe im Eiweiss. (Darstellung der Glucosamino-Mannose), *Biochem. Z.*, 185: 392–399 (1927).

[4] Rimington, C., The Carbohydrate Complex of the Serum Proteins, I and II, *Nature*, 126: 882–883 (1930); *Biochem. J.*, 25: 1062–1071 (1931).

[5] Levene, P. A., and Mori, T., The Carbohydrate Group of Ovomucoid, *J. Biol. Chem.*, 84: 49–61 (1929).

[6] Levene, P. A., and Rothen, A., On the Molecular Size of the Carbohydrates Obtained from Egg Proteins, *J. Biol. Chem.*, 84: 63–68 (1929).

saturation with ammonium sulfate, dialyzed free of ammonium sulfate, dried, and analyzed.

A number of the crude globulins so prepared have been fractionated by adding different amounts of ammonium sulfate to the solution and separating the various fractions which are precipitated at the various concentrations of ammonium sulfate. In the isolation of the globulins by "dissolving" them in neutral salt solution, we are dealing with nothing more nor less than a peptization process, such as is indicated in Fig. 131, where an increase in salt concentration throughout a certain range alters the surface forces in such a manner as to favor dispersion, following which a further increase in salt concentration causes the dehydration or the salting-out of the protein micelles. In this respect, the peptization of protein, as diagrammed in Fig. 131, would differ from the peptization of the silver halide, as diagrammed in Fig. 5, only in the amount of salt required to bring about maximal dispersion.

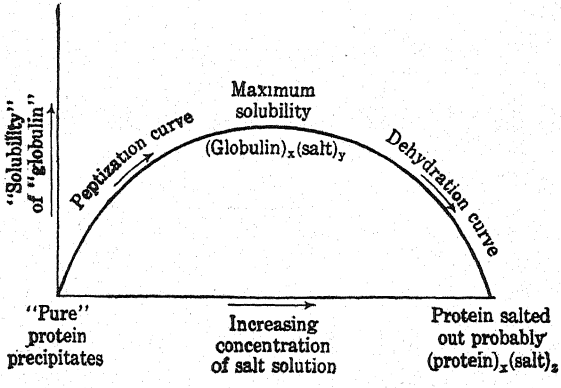


FIG. 131.—A diagrammatic representation of the interrelationships of certain of the factors concerned in protein "solubility."

Subsequent to his classic study of egg albumin Sörensen [7] took up the investigation of serum globulin and concludes that the fractions which have been designated as euglobulin and pseudoglobulin are not capable of being separated as homogeneous entities but that they represent simply arbitrary fractions, the amount and character of the fraction depending on the technic employed for their isolation. We have already seen that Lustig, using non-standard technic, separated four euglobulins and four pseudoglobulins and that Block expresses doubts on the homogeneity of any of the protein fractions which are supposed to characterize the proteins of blood serum.

There are a few vegetable proteins which appear to be homogeneous and which fall in the globulin group. Among these are excelsin from the Brazil nut and pomelin of the orange seed. Re-

[7] Sörensen, S. P. L., Studies on Proteins. VIII. On the Solubility of the Serum Globulins, *Compt. rend. trav. lab. Carlsberg*, Vol. XV, No. 11, 29 pp. (1925).

ferring to Fig. 121, it will be seen that solutions of the various alkali halide salts peptize the Brazil nut protein to almost the same degree, and the same is true for pomelin [8] from the seed of the citrus fruits. Both excelsin and pomelin are crystalline proteins and may be regarded provisionally as true vegetable globulins. Svedberg finds their particle weights to be of the order of 212,000.

Aside from their characteristic peptization behavior toward neutral salt solutions, the chemical properties of the globulins are not greatly different from the chemical properties of many other proteins. Certain of the globulins can be obtained in crystalline form, but here again no exact study of the crystallization process has been made. Certain of the globulins, as already noted, pass into insoluble modifications, the proteans, on standing in contact with pure water or in the presence of hydrogen ions. Little is known in regard to the reason for this transformation, which cannot be regarded as a characteristic property of all globulins, inasmuch as it is not shown by certain compounds classified as globulins.

The vegetable globulins were extensively investigated by Osborne [9] and his students, and his book can be conveniently consulted for specific references. Since his death one of his former associates, D. Breese Jones, has been active in publishing in this field.

The globulins of animal origin which have been studied most extensively are those characteristic of blood serum. It is generally agreed, both from the chemical and the immunological evidence, that the globulin of the blood serum is identical with the globulin which occurs in milk. The albumins of blood serum and lactalbumin, however, are not identical.

The Virus Proteins.—Within the last few years, what appears to be a special class of the globulins has aroused a great deal of interest. These are what have been designated as the virus proteins. In 1935, Stanley [10] isolated a crystalline protein from tobacco plants infected with tobacco-mosaic and after ten successive recrystallizations found that the virus activity resided almost exclusively in this crystalline fraction and that, with the recrystallization, the crystals came to possess a constant and very high virus activity. Later studies by Stanley [11] and co-workers have confirmed the original observa-

[8] Rotha, L. K., and Saunders, F., Studies on Proteins. III. Uniform Solubility of the Protein Fraction of Orange Seed Meal in Solutions of Various Sodium Salts, *J. Am. Chem. Soc.*, 54: 342–345 (1932).

[9] Osborne, T. B., The Vegetable Proteins, Second Edition, Longmans, Green and Company, New York (1924).

[10] Stanley, W. M., Isolation of a Crystalline Protein Possessing the Properties of Tobacco-Mosaic Virus, *Science*, 81: 644–645 (1935).

[11] Stanley, W. M., Chemical Studies on the Virus of Tobacco Mosaic. VI. The Isolation from Diseased Turkish Tobacco Plants of a Crystalline Protein Possessing the Properties of Tobacco-Mosaic Virus, *Phytopathology*, 26: 305–

tions. The protein appears to be isoelectric in the range *p*H 3.3 to 3.49 and to have a sedimentation velocity indicating a micellar weight in the neighborhood of 17,000,000 which, on the assumption of a spherical molecule, would correspond to a diameter of about 35 mμ. It crystallizes in long needles; different preparations show the same virus activity. When small quantities are injected into a plant, symptoms of tobacco virus disease develop and relatively large quantities of the crystalline protein can be isolated from such diseased plants. The virus protein or any protein of similar characteristics is not obtainable from plants which do not show symptoms of tobacco-mosaic. Later studies [12] have indicated that similar virus proteins can be isolated from other organisms showing other virus infections, *e.g.*, the tobacco ring spot virus and tissue of rabbit papillomas. In the isolation of these virus proteins use has been made of the Beams ultracentrifuge (*vide supra*), and all the proteins which have been isolated appear to have particle weights as measured by sedimentation velocities in the range of 20,000,000, with the exception of the bacteriophage protein which Northrop states, "is homogeneous in the ultracentrifuge and has a sedimentation constant of 650×10^{-13} cm. dyne^{-1} sec.$^{-1}$, corresponding to a molecular weight of about 300,000,000." Assuming that the bacteriophage protein has the same specific gravity as egg albumin, a particle weight of 300,000,000 would yield a particle approximately 50 mμ in diameter. Northrop notes that the bacteriophage protein is a nucleoprotein, *i.e.*, nucleic acid is present in the product.

The question naturally arises whether these proteins which have been separated are actually protein molecules or whether they are living organisms, the size of which lies below the limit of microscopic visibility. The author believes that additional data are necessary

320 (1936); VII. An Improved Method for the Preparation of Crystalline Tobacco Mosaic Virus Protein, *J. Biol. Chem.*, 115: 673–678 (1936); VIII. The Isolation of a Crystalline Protein Possessing the Properties of Aucuba Mosaic Virus, *J. Biol. Chem.*, 117: 325–340 (1937); IX. Correlation of Virus Activity and Protein on Centrifugation of Protein from Solution under Various Conditions, *J. Biol. Chem.*, 117: 755–770 (1937); Crystalline Tobacco-Mosaic Virus Protein, *Am. J. Botany*, 24: 59–68 (1937); Wyckoff, R. W. G., Biscoe, J., and Stanley, W. M., An Ultracentrifugal Analysis of the Crystalline Virus Proteins Isolated from Plants Diseased with Different Strains of Tobacco Mosaic Virus, *J. Biol. Chem.*, 117: 57–71 (1937).

[12] Stanley, W. M., and Wyckoff, R. W. G., The Isolation of Tobacco Ring Spot and Other Virus Proteins by Ultracentrifugation, *Science*, 85: 181–183 (1937); Loring, H. S., and Stanley, W. M., Isolation of Crystalline Tobacco Mosaic Virus Protein from Tomato Plants, *J. Biol. Chem.*, 117: 733–754 (1937); Beard, J. W., and Wyckoff, R. W. G., The Isolation of a Homogeneous Heavy Protein from Virus-Induced Rabbit Papillomas, *Science*, 85: 201–202 (1937). *Cf.* also Northrop, J. H., Concentration and Purification of Bacteriophage, *J. Gen. Physiol.*, 21: 335–366 (1938) and Wyckoff, R. W. G., An Ultracentrifugal Analysis of Concentrated Staphylococcus Bacteriophage Preparations, *ibid.*, 21: 367–373 (1938).

before one can eliminate finally the idea of a living organism. If they are "autocatalytic" proteins with the peculiar property of inducing, when injected into living organisms, the production of more proteins of the same type, then they are certainly very unique materials and bridge the gap between living and non-living matter. Autocatalysis, so far as the author is aware, is usually, if not exclusively, a breaking-down process, possibly a chain reaction mechanism where the energy for the reaction is passed on from molecule to molecule, once the chain has been set in motion. A building-up autocatalysis, whereby energy is stored and accumulated, is a special type of autocatalysis which involves such a radical revolution of ideas that the evidence in its favor should be very carefully scrutinized before final acceptance. The fact that the virus proteins sediment with uniform velocity and that the virus activity of the preparation is unchanged after repeated sedimentation is not necessarily evidence of homogeneity. Beams and King[13] centrifuged *Ascaris* eggs under 400,000 × gravity; the eggs lived, had a normal oxygen consumption, and developed normally. When held for 4½ days in a field of 150,000 × gravity, 90 per cent still lived and developed as well as the controls. They conclude that Svedberg's stratification does not take place in the *Ascaris* egg under these enormous gravitational forces and that either protoplasmic structure does not behave as Svedberg's protein systems or else that spatial relationships in protoplasm are not essential to life processes. If no microscope had ever been developed which was powerful enough to render the *Ascaris* eggs visible, one could easily have concluded from these experiments that the *Ascaris* eggs sedimented as uniform-sized protein micelles having an enormous particle weight and that the vital activities characteristic of *Ascaris* eggs were properties of this massive protein "molecule," for the *Ascaris* egg would give the usual protein tests and they would appear as "globular" proteins. The crystallization of the virus protein may be due to a "polarity" of a living organism, since all cells and organisms have definite electrical polarities. Many colonies of the lower organisms assume characteristic shapes and forms, although the colony is composed of a great number of individual organisms. Although the weight of evidence appears to be at the present moment in favor of the virus proteins being non-living biochemical entities or systems (coacervate systems have not been ruled out by any evidence so far presented), one should not ignore the possibility that special types of living organisms may be involved in the phenomena. The recent paper of Wyckoff,[14] who surveys the literature in the field, can be interpreted

[13] Beams, H. W., and King, R. L., Survival of Ascaris Eggs after Centrifuging, *Science*, 84: 138 (1936).

[14] Wyckoff, R. W. G., The Ultracentrifugal Purification and Study of Macromolecular Proteins, *Science*, 86: 92–95 (1937).

just as adequately on the basis of ultramicroscopic living organisms as on the basis of pure virus proteins in spite of the fact that Wyckoff cites the evidence as in favor of the latter viewpoint. As has already been indicated, if these units are proteins, the gap between the living and the non-living has been almost bridged.

III. The Prolamines.—The prolamines or proteins peptized by dilute alcohol are the characteristic proteins of the cereal grains. The name prolamine was given to this group of proteins by Osborne, because, in general, they yield on hydrolysis very large amounts of ammonia (acid amide nitrogen) and relatively large amounts of proline. Gliadin of wheat and rye, zein of maize, and hordein of barley have been most extensively studied. All the cereal grains with the exception of rice contain prolamines. A small amount of an alcohol-soluble protein can be isolated [15] from rice, but it does not show the characteristics of the true prolamines.

The literature of the prolamines, their chemical composition, and certain of their physicochemical properties have been reviewed in a comparative study.[16] There is a great similarity in certain of the physicochemical properties of the proteins of this group, whereas in other properties there is a gradation from gliadin which stands at one end of the series to the prolamines of sorghum and kafir which stand at the other end of the series. Gliadin is peptized by cold 60 to 70 per cent alcohol, whereas the prolamines of kafir and sorghum are not peptized by the cold alcohol but only by hot alcohol. The prolamines are characterized by an almost complete absence of buffering action toward solutions of acid and alkali.

Hoffman and Gortner divide the prolamines into two groups, a wheat group containing the prolamines from *Triticum vulgare, T. spelta, T. durum, T. dicoccum, T. monococcum,* and *Secale cereale,* and a corn group containing the prolamines from *Zea mays, Euchlaena mexicana, Andropogon sorghum,* and *Sorghum vulgare.* The proteins within these two groups have many similar properties, and Lewis and Wells [17] have shown that within the corn group and within the wheat group the prolamines show immunological relationships but that the two groups are distinct when tested by the biological method.

[15] Hoffman, W. F., An Alcohol-Soluble Protein Isolated from Polished Rice, *J. Biol. Chem.*, 66: 501–504 (1925).

[16] Hoffman, W. F., and Gortner, R. A., Physico-Chemical Studies on Proteins. I. The Prolamines—Their Chemical Composition in Relation to Acid and Alkali Binding. Colloid Symposium Monograph, Vol. II, pp. 209–368, Chemical Catalog Company, New York (1925).

[17] Lewis, J. H., and Wells, H. G., The Immunological Properties of Alcohol-Soluble Vegetable Proteins. IX. The Biological Reactions of the Vegetable Proteins, *J. Biol. Chem.*, 66: 37–48 (1925).

Haugaard and Johnson [18] carried out an extensive study on the fractionation of gliadin and conclude that it is a reversible dissociable component system. We have already discussed the peptization of the proteins of wheat flour and have noted that the fractions which we secure by laboratory technics are probably not pre-existent in the wheat berry, or if they are present, they are intimately associated with a larger protein complex, the physicochemical properties of which are not necessarily the additive properties of the fractions which we can isolate.

Mention has been made of the alcohol-soluble protein isolated from milk by Osborne and Wakeman.[19] Although this protein is apparently a distinct protein and is peptized by alcohol, nevertheless it does not contain the large amount of ammonia nitrogen characteristic of most of the prolamines. It is doubtful, therefore, whether it should be classed as a true prolamine, inasmuch as its peptizability by alcohol appears to be the only property which relates it to this class of proteins.

IV. The Glutelins.—These proteins are characterized by not being peptized by neutral solvents but by being peptized by dilute acids and alkalies.

Larmour [20] has collected the literature in regard to these proteins and has added certain new glutelins to the known list. They are found in greatest amount in the cereal grains. There is a progressive change in the chemical analyses of the glutelins in regard to the ammonia nitrogen, arginine nitrogen, and total basic nitrogen content, ranging from glutenin from wheat at one end of the series to oryzenin from rice at the other end of the series, as shown in Table LXIII.

The glutelins of rice varieties and subvarieties [21] show significant differences similar to those found by Larmour for the wheat varieties. All the more recent evidence points to the group of glutelins as being non-homogeneous protein systems capable of fractionation, the characteristics of the fraction depending upon the technic employed.

V. The Albuminoids.—This is a heterogeneous class, generally resistant to chemical reagents and to peptic and tryptic enzymes, and includes such proteins as silk, wool, hair, skin, nails, horn, etc. Cer-

[18] Haugaard, G., and Johnson, A. H., On the Fractionation of Gliadin, *Compt. rend. trav. lab. Carlsberg,* Vol. XVIII, No. 2, 138 pp. (1930).

[19] Osborne, T. B., and Wakeman, A. J., Some New Constituents of Milk III. A New Protein, Soluble in Alcohol, *J. Biol. Chem.*, 33: 243–251 (1918).

[20] Larmour, R. K., A Comparative Study of the Glutelins of the Cereal Grains, *J. Agr. Research,* 35: 1091–1120 (1927).

[21] Tadokoro, T., Nakamura, Y., and Watanabe, S., Differences of the Physico-Chemical Properties of the Protein, Oryzanin, as Found in Glutinous and in Common Rice, *J. Coll. Agr. Hokkaido Imp. Univ.*, 14: 129–169 (1925); Physico-Chemical Studies on the Specificity of Proteins of Different Rice Varieties and Subvarieties, *ibid.*, 16: 73–88 (1926).

TABLE LXIII

VALUES OF ARGININE NITROGEN OF THE VARIOUS GLUTELINS ARRANGED IN ORDER OF MAGNITUDE WITH CORRESPONDING VALUES OF TOTAL BASIC NITROGEN AND AMMONIA NITROGEN

Glutelin	Arginine N, Per Cent	Total Basic N, Per Cent	Ammonia N, Per Cent
Glutenin (Blish)	9.27	18.94	16.5
Zeanin	9.45	18.84	11.32
Glutenin (Larmour)	10.90	18.80	14.78
Duro-glutenin	11.00	21.20	13.25
Hordenin	11.08	20.25	11.38
Monococco-glutenin	11.86	20.16	10.78
Dicocco-glutenin	13.03	24.60	11.06
Spelta-glutenin	13.43	24.44	8.06
Secalinin (average)	13.77	25.69	9.24
Teozeanin	14.15	26.84	10.16
Avenin	15.48	24.37	11.87
Oryzenin	17.95	27.59	8.07

tain of these proteins appear to be made up of relatively few amino acids. Albuminoids do not occur in the vegetable kingdom.

Raw silk can be peptized into two fractions, silk gelatin and silk fibroin, both being classed with the albuminoids. Silk gelatin is peptized by water under slight pressure, or by dilute alkaline solutions, and amounts to 15 to 20 per cent of the weight of the raw silk. Silk fibroin is characterized by its high content of three amino acids, glycine, alanine, and tyrosine, these three accounting for 60 to 70 per cent of the weight of the silk fibroin. Silk, likewise, contains a relatively high percentage of serine (1 to 3 per cent has been actually isolated). Silk gelatin, in contrast to silk fibroin, is extremely low in its glycine content, somewhat higher in its serine content (5.4 to 6.6 per cent has been isolated), but the analyses of silk gelatin account for only 20 to 40 per cent of the amino acids which are present, so that, in general, little can be said in regard to its amino-acid content.

Certain of the albuminoids are of special interest. Thus, *spongin*, the skeletal protein of sponges and coral, contains no tyrosine but instead 3.5-diodo tyrosine or gorgonic acid. The iodine content of spongin ranges from 1 to 1.5 per cent. It is interesting to note that the ancient Greeks considered ground sponges to be specific for goiter. This view, along with many viewpoints of the ancients, was held up to ridicule until modern investigations have shown that certain forms of goiter are due to lack of iodine. The iodine present in 3.5-diodo tyrosine is effective in the prevention of goiter, and dried ground sponges have again been listed as an official remedy in the French Pharmacopœia.

The protein of the coral, *Primnoa lepadifera*, contains 3.5-dibromotyrosine which is one of the few organic compounds of biological origin containing bromine. This amino acid was isolated from the coral protein by Mörner [22] in 1913.

Many of the keratins, notably human hair and wool, contain a very high percentage of cystine, human hair containing as high as 14 per cent of this amino acid. Accordingly, human hair is generally used as the protein for cystine isolation, although wool, which contains considerably less cystine, can be used if necessary. Marston [23] notes that cystine may be the limiting amino acid in wool production. When he supplemented a pasture diet with blood meal which contained 2.7 per cent of cystine, there was a 30 per cent increase in the weight of the wool which was clipped.

Block and Vickery,[24] from a study of several keratins, propose a new definition, *i.e.*: "A keratin is a protein which is resistant to digestion by pepsin and trypsin, which is insoluble in dilute acids and alkalies, in water and in organic solvents, and which, on acid hydrolysis, yields such quantities of histidine, lysine, and arginine that the molecular ratios of these amino acids are respectively approximately 1 : 4 : 12." This definition was based on the study of seven keratins in which the ratios ranged from 1 : 3 : 10 to 1 : 5 : 16. In the second paper human finger nails and cattle horn keratins gave ratios of 1 : 5 : 15 and 1 : 5 : 13, respectively. These are the original studies which later led Block (*loc. cit*) to the conclusion that there was a basic amino acid "anlage" in proteins.

Wilkerson [25] studied the isoelectric point of the keratin from human hair, skin, and nails. He found essentially identical isoelectric points, $p\mathrm{H} = 3.67, 3.70, 3.78$, for these three keratins, and the electrokinetic behavior of the three proteins gave curves which were essentially superimposable.

Elastin, the principal protein of tendons, cartilage, bones, and connective tissue, is noted for its high content of glycine and leucine,

[22] Mörner, C. T., Zur Kenntnis der organischen Gerüstsubstanz des Anthozoënskeletts. IV. Isolierung und Identifizierung der Bromgorgosäure, *Z. physiol. Chem.*, 88: 138–154 (1913).

[23] Marston, H. R., The Production of Wool Fleece by Merino Sheep, *Australian J. Exptl. Biol. and Med. Sci.*, 9: 235–246 (1932); *cf.* also Marston, H. R., Studies on the Relationship Between Nutrition and Wool Production of Merino Sheep, I and II, *J. Agr. Science*, 25: 103–112, 113–131 (1935).

[24] Block, R. J., and Vickery, H. B., The Basic Amino Acids of Proteins. A Chemical Relationship between Various Keratins, *J. Biol. Chem.*, 93: 113–117 (1931); Block, R. J., The Basic Amino Acids of Keratin. The Basic Amino Acid Content of Human Finger Nails and Cattle Horn, *J. Biol. Chem.*, 104: 339–341 (1934).

[25] Wilkerson, V. A., The Chemistry of Human Epidermis, I and II, *J. Biol. Chem.*, 107: 377–381 (1934); *ibid.*, 112: 329–335 (1935).

these two amino acids making up approximately half of the total weight of the protein.

Gelatin, obtained by extracting hides, bones, etc., with hot water, is interesting because of the absence, or almost complete absence, of three amino acids, trytophane, cystine, and tyrosine. A fourth amino acid, histidine, is present only in exceedingly small amounts. The proline and glycine contents are high, 7.7 and 6.5 per cent, respectively.

Since gelatin is derived from collagen, it should probably be classed as a derived protein but is usually included in the group of albuminoids. Probably gelatin has been made the basis of more physicochemical studies than any other protein because of its ready availability and the fact that its solutions undergo sol $\rightleftharpoons$ gel transformations with changes in temperature. In many respects it is a unique protein, and its sols and gels show remarkable properties. For example, when in the sol state it shows a specific optical rotation of approximately 160°, with practically no change in optical rotation when in the sol state (40° C.) over the range *p*H 1–12. On gelling, the specific optical rotation increases over 200 per cent, with marked maxima between *p*H 1–2, *p*H 4–5, *p*H 7–9, and a marked minimum between *p*H 2–3.[26] Most gels are not sufficiently optically clear to permit of polarimetric studies. Accordingly there has been doubt as to whether the enormous increase in optical rotation exhibited by the gelling of gelatin is a characteristic of all gels or is specific for gelatin gels. Recent studies in the author's laboratories have shown that zein gels (in glycol monomethyl ether) have the same specific optical rotation as the sols from which the gels were derived. Accordingly the change of optical rotation of gelatin in passing from the sol to gel state would appear to be a unique property of a gelatin system and probably is associated with binding of water by the protein micelles.

Gelatin sols and gels show enormous changes in viscosity with changes in hydrogen-ion concentration, and these viscosity changes in a large degree parallel the changes in optical rotation.[27] Again the phenomena appear to be associated with the binding of water on the polar groups associated with the asymmetric carbon atom.

Gelatin systems show marked hysteresis, and accordingly the physicochemical properties of the system vary with time and are in a very considerable measure dependent upon the source of the gelatin used and the methods employed in the preparation of the sols and gels. Ultracentrifugal studies have shown gelatin to be non-homo-

[26] Kraemer, E. O., Observations of the Colloidal Behavior of Aqueous Gelatin Systems, Colloid Symposium Monograph, Vol. IV, pp. 102–121, Chemical Catalog Company, New York, 1926.

[27] Bogue, R. H., and O'Connell, M. T., The Influences of Hydrogen Ion Concentration on the Optical Rotation of Gelatin, *J. Am. Chem. Soc.*, 47: 1694–1697 (1925).

geneous in particle size, and, because of this non-homogeneity and the great variation which exists in the commercial preparations, it is not surprising that the literature contains many conflicting observations on the physicochemical properties of gelatin systems.

VI. THE HISTONES.—These are basic proteins, characterized by a relatively high proportion of diamino acids and by the fact that at least some of them form salts with the strong mineral acids. Their nitrogen content ranges in the neighborhood of 17 to 20 per cent. They combine readily with nuclein or nucleic acid, probably as coacervate systems, and are the characteristic proteins of tissues rich in cells, such as glandular tissues. It has been suggested that the histones represent the union of a protamine with some other protein. The histones which have been most extensively investigated are the globins from the erythrocytes of blood, particularly from the blood of birds, where the histone occurs combined with the non-protein radical, hemin, and the histone of the thymus gland, where it occurs combined with nucleic acid. Histones also occur in the unripe sperm of fish and sometimes in the ripe sperm of some vertebrates and some invertebrates. They are, in general, characterized by a high arginine content.

VII. THE PROTAMINES.—The protamines are the characteristic proteins of ripe sperm cells and are apparently the proteins associated with nucleic acid in *chromatin*. Here again we are probably dealing with coacervate systems, inasmuch as these proteins are so basic that in the free state they will blue litmus paper and have an isoelectric point well on the alkaline side of neutrality. They are therefore positively charged in the *p*H range where most proteins carry a negative charge, which accounts for the great ease with which they form coacervates with proteins, nucleic acid, arabic acid, and any other negatively charged lyophilic colloid. The nitrogen content is high, ranging from 25 to 30 per cent of the weight of the protein. They are sulfur-free. They are easily soluble in water, insoluble in alcohol and ether, and do not coagulate, and do not diffuse through parchment membrane. With mineral acids they form stable compounds, many of which are crystalline, and the free protamine will absorb carbon dioxide from the air, forming a more or less stable protamine carbonate. Double salts of constant composition are formed with platinic chloride and auric chloride. They are not digested by pepsin but are hydrolyzed by trypsin and erepsin.

Kossel [28] and his associates have made the most extensive investigations of the protamines. He classifies the protamines into the *monoprotamines*, the *diprotamines*, and the *triprotamines*.

[28] Kossel, A., The Protamines and Histones, translated by W. V. Thorpe, Longmans, Green and Company, New York (1928); *cf.* also *Z. physiol. Chem.*, 44: 347–352 (1905); *ibid.*, 49: 301–321 (1906).

The monoprotamines contain only arginine in the diamino-acid fraction. Within the monoprotamine group, he has two subclasses which he designates as the a_2m–a_4m class and the am_2 class where the a represents an arginine residue and the m represents the residue of a monoamino acid. In the a_2m–a_4m class he lists the protamines: salmine, coregonine, truttine, salveline, clupeine, scrombrine, esocine, alalongine, thynnine, and ancylodine. The am_2 class has only one representative, cyclopterine.

Kossel's diprotamines contain two diamino acids, and again there are two subclasses: a *percine* class having the general formula $(ah)_2m$, where arginine and histidine are present; and a *cyprinine* class, $(al)m_x$, where arginine and lysine are the diamino acids.

The third major group is the triprotamines, $(ahl)_2m$, containing all three of the hexone bases, with sturine as the only representative so far characterized.

The two protamines which have been most completely characterized are salmine and clupeine, which have recently been reinvestigated.[29]

Salmine is the protamine from ripe salmon sperm. All evidence indicates that it is a polypeptide containing 21 amino-acid residues and 20 peptide linkages. It contains 14 molecules of arginine, 3 molecules of proline, 3 molecules of serine, and 1 molecule of valine. It contains no free amino group, proline being at the beginning of the polypeptide chain, so that what would have been normally a free amino group is the imino group of proline. At the carboxyl end of the polypeptide chain there are apparently 2 molecules of arginine. It will be noted that there are 2 arginine residues for each monoamino-acid residue, and Waldschmidt-Leitz suggests that it is composed of a series of tripeptides linked together possibly in the following arrangement: M-D-D-M-D-D-M-D-D-M-D-D-M-D-D-M-D-D-M-D-D-M-D-D, where M represents a monoamino-acid residue, and D an arginine residue. Such a structure would have a molecular weight of 2,855, which agrees well with sedimentation velocity measurements carried out in Svedberg's laboratory and with the other physicochemical properties of salmine solutions.

Waldschmidt-Leitz isolated from pancreas extract a new specific enzyme, protaminase, which acts exclusively on protamines to split off arginine when the carboxyl group of arginine is free. When this protaminase acts upon salmine, the 2 terminal arginine molecules are split off leaving a residue, *salman*, containing 7 monoamino acids and 12 arginine residues. It is on the basis of this observation that Waldschmidt-Leitz concludes that not more than 2 arginine residues are conjugated together in the structure.

[29] Waldschmidt-Leitz, E., Ziegler, F., Schäffner, A., and Weil, L., Über die Struktur der Protamine I. Protaminase und die Produkte ihrer Einwirkung auf Clupein und Salmin, *Z. physiol. Chem.*, 197: 219–236 (1931).

Clupeine is the protamine of herring sperm. It contains 15 amino-acid residues and 14 peptide linkages. The amino-acid composition is 10 molecules of arginine, 2 molecules of serine, and 1 each of proline, alanine, and valine, with the molecular weight of 2,021. Again the general arrangement of the formula noted for salmine appears to apply to clupeine, with proline at one end of the chain, thus providing an imino rather than an amino group and with 2 residues of arginine at the carboxyl end of the chain. Protaminase splits off these 2 molecules of arginine leaving a tredecapeptide, clupean, corresponding to the salman of salmine.

Rasmussen [30] working in Sörensen's laboratories, expresses a doubt as to the homogeneity of clupeine as ordinarily prepared. In a series of fractionations he secured preparations in which the arginine N ranged from 87.2 per cent to 91.7 per cent of the total N and concludes that the clupeine molecule cannot be built up exclusively of a series of tripeptides each containing 2 molecules of arginine to 1 molecule of a monoamino acid, since such a system would have 88.9 per cent of the total N as arginine N. In a later study [31] dealing with the electrometric titration of clupeine, the belief is expressed that it has a molecular weight lying between 4,000 and 4,100. This would necessitate doubling the molecule proposed by Waldschmidt-Leitz.

Scombrine, from mackerel sperm, is probably the simplest protamine, apparently containing only three amino acids, arginine 88.8 per cent of the total N, proline 3.8 per cent of the total N, the remainder being alanine, although there is a possibility that some other amino acid may be present in the alanine fraction. Kossel notes that the absence of serine distinguishes scombrine from salmine and clupeine.

Percine is apparently the characteristic protamine of the yellow perch, *Perca flavescens,* and the pike perch, *Stizostedium vitreum.* It contains 78.1 per cent of its total N as arginine N, 5.6 per cent as histidine N, and the balance as monoamino N. The monoamino acids have not been identified.

Sturine, from sturgeon sperm, contains all three of the diamino acids in the proportions of arginine 58.2 per cent, histidine 12.9 per cent, lysine 12.9 per cent, the three totaling 84 per cent of the protein molecule. The remaining amino acids are apparently exclusively leucine and alanine.

When protamines are injected intravenously,[32] they cause a rapid

[30] Rasmussen, K. E., Investigations of Clupein I. Preparation and Fractionation of Clupein, *Compt. rend. trav. lab. Carlsberg,* Vol. XX, No. 6, 20 pp., (1934).

[31] Rasmussen, K. E., and Linderström-Lang, K., Investigations of Clupein II. Electrometric Titration of Clupein, *Compt. rend. trav. lab. Carlsberg,* Vol. XX, No. 10, 36 pp. (1935).

[32] Thompson, W. H., Die physiologische Wirkung der Protamine und ihrer Spaltungsprodukte, *Z. physiol. Chem.,* 29: 1–19 (1900).

fall in blood pressure and decreased respiration, death ensuing at even low dosages. The fatal dose of clupeine, injected intravenously, lies between 0.15 and 0.18 gram for a 10-kg. dog. The lethal dosage of sturine lies between 0.20 and 0.25 gram for a dog of similar weight. With a non-lethal dose, the initial reaction is over after a 30-minute period, but a second dose causes the same symptoms, indicating that no appreciable degree of immunity is conferred. The intravenous injection causes a marked delay in blood-coagulation time, which may be as much as 36 hours. The same effect is produced on blood coagulation *in vitro*. Leucocytes are rapidly destroyed by the intravenous injection of protamines. A sublethal injection of sturine reduced the leucocyte count from a normal count of 28,281 to 1,875 after a single injection and to 781 after a second injection. The diamino acids alone did not produce the above physiological symptoms, nor did a mixture of all the hydrolytic products of the protamine. Thompson accordingly concludes that the toxic action is due to the specific groupings in the molecule.

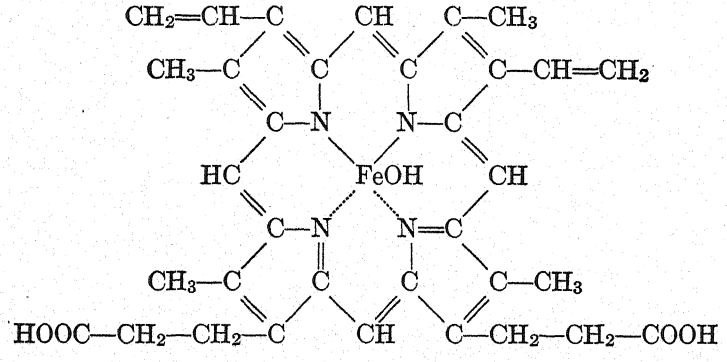
Recently Hagedorn [33] and his associates have combined protamines with insulin to form a "protamine insulinate" for use in the treatment of diabetics. They note that the protamine insulinate is relatively insoluble, it is absorbed slowly, and the insulin thus becomes available to the patient in small amounts over a relatively long period of time. When the free insulin is injected, there is a sharp reduction in blood sugar followed a few hours later by a similar rapid rise. With the injection of protamine insulinate the sharp reduction is avoided and the risk of hypoglycemia is thus greatly reduced. The reduction of blood sugar is slow and regular, and the regain of blood sugar likewise is slow and regular. Larger quantities of insulin can be injected, and thus the spacing between injections can be prolonged. The compound of protamine with insulin is probably a coacervate and contains about 1 part of protamine to 10 parts of insulin. One unit of insulin approximates 0.04 mg. Accordingly with an injection of even 25 units of insulin in the form of protamine insulinate there would be an insignificant amount of protamine injected, which probably accounts for the fact that no untoward symptoms have been observed which could be attributed to the protamine. Hagedorn found that the protamine insulinates, where clupeine, scombrine, or salmine was used, were too insoluble for best results. The clupeine insulin had its minimum solubility at *p*H 6.3, the scombrine and salmine compounds at *p*H 7.3. At these minimum solubilities, the solubility of the compound was of the order of that of barium sulfate. He accordingly used cyclopterine

[33] Hagedorn, H. C., Jensen, B. N., Krarup, N. B., and Wodstrup, I., Protamine Insulinate, *J. Am. Med. Assoc.*, 106: 177–180 (1936); *cf.* also Root, H. F., White, P., Marble, A., and Stotz, E. H., Clinical Experience with Protamine Insulinate, *ibid.*, 106: 180–183 (1936).

from *Salmo iridius*, which gave a compound with the desired solubility. Since the original observations of Hagedorn, protamine insulinate has been widely adopted in medical practice.

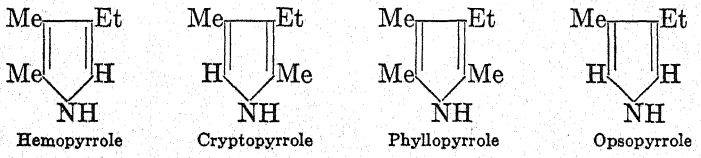
VIII. The Chromoproteins. *Hemoglobins.*—The chromoproteins include the *hemoglobins*, characteristic of the red blood cells of the mammals. The associated radical is hematin or "protohematin," which has the formula $C_{34}H_{32}O_4N_4FeOH$, united with a histone, globin. Globin makes up about 94 per cent and hematin about 6 per cent of the hemoglobin molecule.

Hans Fischer, in a series of brilliant researches which led to the award of the Nobel prize in 1931, finally succeeded in synthesizing the prosthetic group, so that its structure is definitely known. The synthetic product is identical in all respects with the natural product and has the formula [34]



Hematin (H. Fischer)

By drastic reductive fission with hydriodic acid, hematin gives rise to four hemopyrroles having the following formulas:



Hemopyrrole | Cryptopyrrole | Phyllopyrrole | Opsopyrrole

The iron is held in the hematin molecule, probably in a chellate linkage, in non-ionizable form, and hematin is readily converted into the corresponding hemin, $C_{34}H_{32}O_4N_4FeCl$, by treatment with hydrochloric acid or with acetic acid and sodium chloride. By various chemical reagents hematin can be broken down into a series of por-

[34] Fischer, H., and Zeile, K., Synthese des Hämatoporphyrins, Protoporphyrins und Hämins XXII. Mitteilung über Porphyrin-Synthesen, *Ann.*, 468: 98–116 (1929).

phyrins, the more important of which are shown in Table LXIV. On breaking down in the animal body, hematin gives rise to the bile pigments which are also the pigments of gallstones, the shells of birds' eggs, etc.

The oxygen-carrying capacity of the blood is associated with the hematin and presumably is an addition of oxygen to the iron atom,[35] in which one atom of iron in the hematin unites with one molecule of oxygen. The only function of the hemoglobin in the blood stream is to carry this molecule of oxygen from the lungs to the various cells and tissues where the oxygen pressure is lower, under which condition the oxygen is released from the hemoglobin and diffuses through the capillary walls into the tissue cells where it is utilized for respiration. Hemoglobin itself has no catalytic action in stimulating respiration.

The iron content of hemoglobins from different animals varies from 0.33 to 0.51 per cent according to the species, and based on the iron content hemoglobin would have a minimum molecular weight of from 16,000 to 17,000. Svedberg, using the ultracentrifuge, finds a particle weight of about 68,000, and this value has been confirmed by other technics. Apparently, therefore, four hematin-globin units are united in the hemoglobin micelle.

The histone, globin, has its isoelectric point at *p*H 7.5.[36] The globin can be separated from the hematin and later recombined to form a

TABLE LXIV[37]

THE MORE IMPORTANT PORPHYRINS DERIVED FROM HEMATIN

		Isomerides		Side Chains
		Possible	Synthesized (by H. Fischer)	
Hemin	$C_{34}H_{32}O_4N_4FeCl$...	15	2	4Me 2X* 2CH : CH_2
Protoporphyrin	$C_{34}H_{34}O_4N_4$.....	15	2	4Me 2X 2CH : CH_2
Hematoporphyrin	$C_{34}H_{38}O_6N_4$.....	15	2	4Me 2X 2CH(OH)Me
Mesoporphyrin	$C_{34}H_{38}O_4N_4$.....	15	12	4Me 2X 2Et
Aetioporphyrin	$C_{32}H_{38}N_4$.....	4	4	4Me .. 4Et
Deuteroporphyrin	$C_{30}H_{30}O_4N_4$.....	15	3	4Me 2X ...
Deuterohemin	$C_{30}H_{28}O_4N_4FeCl$...	15	2	4Me 2X ...

* X = $-CH_2CH_2COOH$.

[35] Anson, M. L., and Mirsky, A. E., Hemoglobin, the Heme Pigments and Cellular Respiration, *Physiol. Rev.*, **10**: 506–546 (1930).

[36] Roche, J., Recherches sur la globine, *Compt. rend. trav. lab. Carlsberg*, Vol. XVIII, No. 4, 34 pp. (1930).

[37] From King, H., Pyrrol Pigments, *Ann. Repts. Chem. Soc. London*, 29: 209–219 (1932).

hemoglobin which has the properties of the natural hemoglobin. Apparently the combination of the hematin and globin to form hemoglobin is not a direct union of carbon with carbon but is more like that of an acid with a base. Hill and Holden [38] effected the first resynthesis. Herzog [39] synthesized a number of hemoglobins by coupling hematin with globins derived from various animal sources. *The crystallographic properties of the synthetic hemoglobins were identical with those of the natural hemoglobins, the crystal form depending upon the biological source of the globin which was used.*

Other bases much more simple than globin, such for example as pyridine and nicotine, can be combined with hematin to form hemopyridine, hemonicotine, etc. Although these have properties which are different from hemoglobin, nevertheless they show a number of reactions which simulate the reactions of hemoglobin and which presumably reflect the properties of the hematin nucleus.

If one compares the formula of hematin with that of chlorophyll (*vide infra*), it will be noted that there is a striking similarity, indicating that the most important pigment of the plant kingdom and the most important pigment of the higher animals have much in common and are actually modifications of the same structure.

Warburg paid particular attention to the role of iron in the process of oxidation. In a summary paper he [40] notes that *hematin*, the nucleus of hemoglobin, not only is present in the form of hemoglobin in animal cells and tissues but that *it is likewise present in all plant and animal cells*, including the bacteria and the yeasts, the only difference being that in the red blood cells it is combined with the protein, globin, whereas in other cells it may exist either free, or combined with other compounds.

It is well known that carbon monoxide acts as a toxic gas because of the formation of carbon-monoxide-hemoglobin in which the carbon monoxide is more firmly held on the iron atom than is molecular oxygen. Warburg points out that in experiments conducted by Haldane, where mice were exposed to an atmosphere of carbon monoxide, the carbon monoxide displaced the oxygen in hemoglobin and the mice died for lack of oxygen. When, however, such mice before death were placed in an atmosphere of higher oxygen content, the total hemoglobin still remained combined with carbon monoxide but the dissolved oxygen in the blood plasma increased tenfold. In this case, the mice did not

[38] Hill, R., and Holden, H. F., The Preparation and Some Properties of the Globin of Oxyhaemoglobin, *Biochem. J.*, 20: 1326–1339 (1926).

[39] Herzog, A., Über die prosthetische Gruppe des Blutfarbstoffes, deren kristallinische Isolierung und deren Synthese mit nativem Globin zu Hämoglobin, *Biochem. Z.*, 264: 412–430 (1933).

[40] Warburg, O., The Chemical Constitution of Respiration Ferment, *Science*, 68: 437–443 (1928), translated from the German, *Naturwissenschaften*, 16: 345–350 (1928).

die because the oxygen dissolved in the blood plasma was sufficient to supply the tissues for the process of respiration. Warburg points out that this experiment indicates that carbon monoxide does not affect the respiration of the cells even when the hemoglobin is completely saturated with carbon monoxide.

He then proceeds to the theory that the enzyme which is responsible for the respiration process is hematin or a hematin derivative. He notes that the equilibrium

$$\text{oxyhemoglobin} + \text{carbon monoxide} \rightleftharpoons \text{hemoglobin-carbon monoxide} + \text{oxygen}$$

is shifted to the left when the system is exposed to light. In other words, the hemoglobin is regenerated when carbon-monoxide-hemoglobin is illuminated. He finds a similar effect of carbon monoxide upon respiration when the experiments are conducted in the dark and in the light. In the dark the respiration of cells is inhibited by carbon monoxide, the inhibition diminishing and disappearing rapidly when cells are illuminated. He notes that the intensity of light required to effect this photochemical change, so far as the respiration enzyme is concerned, is only one ten-thousandth of the intensity of sunlight.

Warburg notes that the respiration enzyme has the three characteristic properties of hemoglobin. It reacts reversibly with carbon monoxide and oxygen; the carbon monoxide and oxygen show the same distribution coefficient,

$$\frac{HbO_2}{HbCO} \cdot \frac{CO}{O_2} = K$$

and the combination with carbon monoxide is sensitive to light. The difference between hemoglobin and the respiration enzyme is noted to be that the respiration enzyme acts as a catalyst, whereas hemoglobin does not. Accordingly, Warburg believes that the respiration enzyme approximates the properties of free hematin more closely than the properties of hemoglobin. Free hematin catalytically oxidizes cysteine in aqueous solution to cystine, but this catalysis is inhibited by carbon monoxide. Here again the system is only moderately sensitive to light. If, however, the hematin is combined with pyridine or nicotine, very powerful catalysts were obtained capable of transferring 2,000 molecules of oxygen per molecule of hemonicotine or hemopyridine to the cysteine, and the carbon monoxide compounds of these hematin derivatives had the same remarkable reaction toward light, the carbon monoxide being liberated by light of one ten-thousandth of the intensity of sunlight.

Warburg then studied the effect of light of various wave lengths on cell respiration in the presence of certain amounts of carbon monoxide and secured what are essentially absorption spectrum curves, as meas-

ured with a photometer, for carbon monoxide hemonicotine and his hypothetical carbon monoxide respiration enzyme, using the respiration of living cells in the latter instance. *He finds the two absorption curves to be essentially identical,* the only difference being that the entire curve is shifted approximately 20 mμ toward the red end of the spectrum, as shown in Fig. 132. He notes that curve *B*, showing the characteristic shift toward the red end of the spectrum, is characteristic of the spectrum of a solid compound, rather than of a dissolved compound, that when an absorption spectrum is taken of material in the solid state, there is regularly a shift of approximately 20 mμ toward the red end of the spectrum as compared to the curve of the material

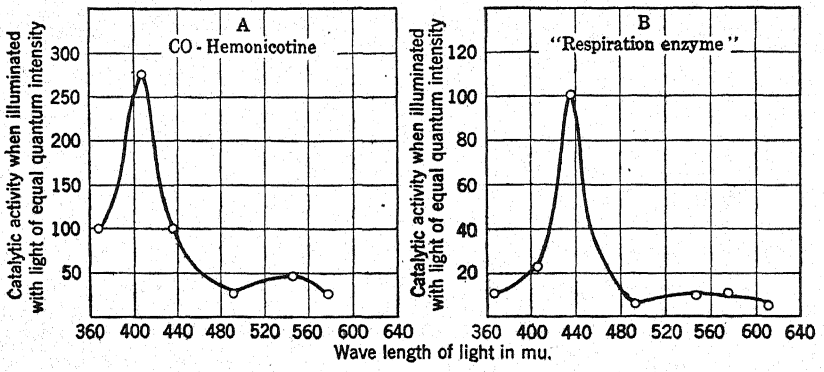


FIG. 132.—Showing the similarity in absorption spectra of carbon monoxide hemonicotine and the respiration enzyme (curve *B*), as measured by studying their catalytic activity when illuminated with light of pure wave length and equal quanta intensity. (Data of Warburg.)

in solution. He accordingly suggests that *the respiratory enzyme is hematin combined with some as yet unknown base and that it is not in solution in the cell but is imbedded in* (*adsorbed upon* [*?*]) *the surfaces of the solid cellular components.*

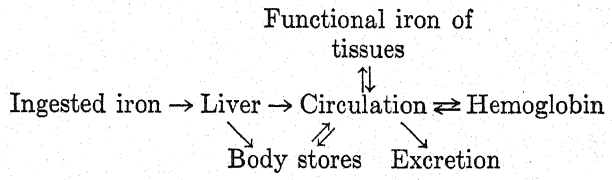
These observations of Warburg have been confirmed by numerous investigators, and there is no question at the present time but that the respiration enzyme of all cells and tissues is a hematin derivative. Recently it has been demonstrated [41] that catalase is likewise a hematin-containing enzyme, the porphyrin of which is the same as the porphyrin of the blood pigment. The iron, however, is in the ferric state. The catalase, protohematin, shows maximum light extinction at 409 mμ with other bands at 505, 540, and 622 mμ.

One molecule of cyanide reacts with the globin of hemoglobin, first to form an addition product, and then, with the addition of a second

[41] Stern, K. G., Spectroscopy of Catalase, *J. Gen. Physiol.*, 20: 631–648 (1937).

molecule of cyanide, the globin residue is split off leaving a hematin-dicyanide residue.[42]

The synthesis of hematin and accordingly the synthesis of hemoglobin in the animal body is not as yet fully elucidated. Synthesis apparently takes place primarily in the bone marrow, in the liver, and in the spleen. On a diet deficient in iron, nutritional anemia develops. Rather interestingly, iron is not the only mineral element essential in the biological synthesis of hemoglobin, for both copper[43] and manganese are in some way involved in the vital synthetic processes, and in the absence of copper and manganese nutritional anemia still appears. Still more recent observations[44] indicate that cobalt is likewise an essential element concerned in the synthesis of hemoglobin. Neither copper, manganese, nor cobalt appear in the final product, and their role in the synthetic mechanism still remains to be elucidated. Josephs[45] believes that the role of copper is in mobilizing tissue iron to form hemoglobin or else that the copper prevents the tissues from the storing of tissue iron. His scheme of iron distribution within the body is indicated in the following diagram:



[42] Anson, M. L., and Mirsky, A. E., The Reactions of Cyanide with Globin Hemochromogen, *J. Gen. Physiol.*, 14: 43–54 (1930).

[43] Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A., Iron in Nutrition. VII. Copper as a Supplement to Iron for Hemoglobin Building in the Rat, *J. Biol. Chem.*, 77: 797–812 (1928); Titus, R. W., and Cave, H. W., Manganese as a Factor in Hemoglobin Building, *Science*, 68: 410 (1928); Mitchell, H. S., and Miller, Lila, Inorganic Elements of Spinach in Treatment of Nutritional Anemia, *Proc. Soc. Exptl. Biol. Med.*, 26: 835–836 (1929); Becker, R. B., Neal, W. M., and Shealy, A. L., Salt Sick—Its Cause and Prevention, Bull. 231, Florida Agr. Exp. Sta.. June, 1931.

[44] Filmer, J. F., Enzootic Marasmus of Cattle and Sheep. Preliminary Report, Having Special Reference to Iron and Liver Therapy, *Australian Vet. J.*, 9: 163–179 (1933); Filmer, J. F., and Underwood, E. J., Enzootic Marasmus. Treatment with Limonite Fractions, *ibid.*. 10: 83–87 (1934); Underwood, E. J., Enzootic Marasmus. Iron Content of Liver, Kidney and Spleen, *ibid.*, 10: 87–92 (1934); Underwood, E. J., and Filmer, J. F., Enzootic Marasmus. The Determination of the Biologically Potent Element (Cobalt) in Limonite, *ibid.*, 11: 84–92 (1935); Askew, H. O., and Dixon, J. K., Influence of Cobalt Top-dressing on the Cobalt Status of Pasture Plants, *New Zealand J. Sci. and Tech.*, 18: 688–693 (1937); Cobalt Status of Animal Organs from South Island (N. Z.) Drench Experiments, *ibid.*, 18: 707–716 (1937); Kidson, E. B., Cobalt Status of New Zealand Soils, *ibid.*, 18: 694–707 (1937).

[45] Josephs, H. W., Studies on Iron Metabolism and the Influence of Copper, *J. Biol. Chem.*, 96: 559–571 (1932).

It seems probable that the role of manganese and cobalt is likewise of a catalytic nature.

We have already noted that the prosthetic group in hemoglobin is closely allied to the porphyrin of chlorophyll. *Copper and manganese are likewise essential for the development of the chlorophyll molecule.*[46] So far as the author is aware, cobalt has not as yet been demonstrated to be essential for chlorophyll formation.

The Hemocyanins.—The blood of the cephalopods, such as the squid and octopus, and of the Crustacea, such as lobsters, and of the Mollusca, does not contain hemoglobin but rather a chromoprotein known as *hemocyanin*, in which the iron is replaced by *copper*, the copper content ranging from 0.33 to 0.38 per cent. The color change of oxidized to reduced hemocyanin is from light blue to colorless. A Van Slyke analysis[47] of the hemocyanin of the crab (*Limulus polyphemus*) indicates 31.3 per cent of the total nitrogen to be present in the form of arginine, histidine, and lysine. The prosthetic group, if one is present, has not been definitely identified. Certain workers have claimed the isolation of a pigmented group which gives the pyrrole reaction and which they believe to be a porphyrin. However, Conant[48] *et al.*, studying the hemocyanin of *Limulus*, separated a "complex cupric salt of a polypeptide composed of 3 molecules of serine, 1 of leucine, 1 of tyrosine, and some sulfur-containing compound," which they believe is the prosthetic group and which contains all the copper in the hemocyanin. The question naturally arises whether this material which they isolated is actually a prosthetic group or whether it is a fragment of the larger protein molecule, perhaps of the original hemocyanin itself. The author believes that the weight of evidence is against the hemocyanins carrying a true porphyrin prosthetic group.

The hemocyanins which have been investigated by Svedberg have given particle weights ranging from approximately 2,000,000 for those derived from *Limulus* and *Octopus* to 5,000,000 for the hemocyanin of *Helix pomata*. The oxygen-carrying capacity[49] of hemocyanin is only

[46] Sommer, A. L., Copper as an Essential for Plant Growth, *Plant Physiol.*, 6: 339–345 (1931); Oserkowsky, J., and Thomas, H. E., Exanthema in Pears and Its Relation to Copper Deficiency, *Science*, 78: 315–316 (1933); Orth, O. S., Wickwire, G. C., and Burge, W. E., Copper in Relation to Chlorophyll and Hemoglobin Formation, *Science*, 79: 33–34 (1934).

[47] Alsberg, C. L., and Clark, E. D., The Haemocyanin of Limulus Polyphemus, *J. Biol. Chem.*, 8: 1–8 (1910).

[48] Conant, J. B., Dersch, F., and Mydans, W. E., The Prosthetic Group of *Limulus Hemocyanin*, *J. Biol. Chem.*, 107: 755–766 (1934).

[49] Stedman, Ellen, and Stedman, Edgar, Haemocyanin, Pt. I. The Dissociation Curves of the Oxyhaemocyanin in the Blood of Some Decapod Crustacea, *Biochem. J.*, 19: 544–551 (1925); Pt. II. The Influence of Hydrogen Ion Concentration on the Dissociation Curve of the Oxyhaemocyanin from the Blood of the Common Lobster (*Homarus vulgaris*), *Biochem. J.*, 20: 938–948 (1926); Pt. III. The In-

approximately one-fourth that of hemoglobin. The fact that the respiratory pigment in these lower forms of animal life contains copper rather than iron and that the copper-containing compound is less efficient than hemoglobin in its vital functions, combined with the fact that copper is essential for the formation of hemoglobin, leads almost inevitably to the speculation whether the role of copper in the formation of hemoglobin may not be a vestigial remnant of a vital mechanism carried over in the processes of organic evolution. In the early stages of embryonic growth of the pig [50] so much copper is present in the embryo that the ash is colored a characteristic blue. No studies have as yet been made to see whether or not hemocyanin may not be present in the very early stages of mammalian embryonic growth.

Other Blood Pigments.—Griffith [51] isolated from the tropical mussel, *Pinna squamosa,* a compound which contained neither iron nor copper but rather manganese which he reports as present to the extent of 0.81 per cent. He believed this to be the respiratory pigment and named it *pinnaglobin,* adding that its color change from the oxidized to the reduced condition was from brown to colorless. A reinvestigation by Henze [52] indicates that the manganese-containing compound is not derived from the blood fluids but rather from the pericardial fluid. The pigment isolated contained only 3.8 per cent of nitrogen; it was not a protein; it did not have respiratory properties, although it contained approximately 0.6 per cent manganese in a stable linkage. Henze concludes that "pinnaglobin" does not exist.

Perhaps compounds somewhat similar to this manganese compound are those pigments containing vanadium [53] which occur in *ascidians* and the *tunicates.* The vanadium compound may be a chromoprotein. In *Phallusia mamillata* the blood pigment contains 10 per cent of vanadium. It is present in the reduced form; it is not an oxygen carrier but is rather a powerful reducing agent. It is suggested that it may be a photosynthetic catalyst converting carbon dioxide into carbohydrates. In the tunicates the blood ash may contain as much

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fluence of Hydrogen Ion Concentration on the Dissociation Curve of the Oxyhaemocyanin from the Blood of the Edible Crab (*Cancer pagurus*), *Biochem. J.,* 20: 949–956 (1926).

[50] Wilkerson, V. A., and Gortner, R. A., *loc. cit. Cf.* also Wilkerson, V. A., The Chemistry of Embryonic Growth IV. The Requirement of the Pig Embryo for Copper, *J. Biol. Chem.,* 104: 541–546 (1934).

[51] Griffith, A. B., Sur la composition de la pinnaglobine; une nouvelle globuline, *Compt. rend.,* 114: 840–842 (1892).

[52] Henze, M., Über das sogenannte "Pinnaglobin," *Z. physiol. Chem.,* 162: 136–138 (1926).

[53] Henze, M., Über das Vanadiumchromogen des Ascidienblutes, *Z. physiol. Chem.,* 213: 125–135 (1932); and Cantacuzène, J., and Tchekirian, A., Sur la présence de vanadium chez certains Tuniciers, *Compt. rend.,* 195: 846–849 (1932).

as 15.4 per cent of vanadium. We have already noted [54] the presence of free sulfuric acid in the corpuscle fluid of *Chelyosoma siboja* reaching the high concentration of 0.88 *N*. Both the body fluid and the corpuscle fluid contain vanadium pigment, the ash of the blood being largely vanadium oxide. Whether or not porphyrins are present in these vanadium compounds still remains to be ascertained.

Chlorocruorin is a green oxygen-containing pigment which can be obtained in crystalline form from the blood of polychaete worms. The prosthetic group appears to be a porphyrin, but it is not hematin. The red pigment occurring in the blood of a number of worms is known as *hemoerythrin* and appears to have a porphyrin residue but does not yield the mammalian hematin. *Erythrocruorins*, the red respiratory pigments of a number of the lower forms, such as *Daphnia* and the earthworm, together with the chlorocruorins, show much similarity to the hemoglobin of mammalian blood. The porphyrin contains iron, and the ratio of oxygen to iron in the oxygen-carrying capacity of the blood is the same as hemoglobin. Svedberg has measured the particle weight of a number of erythrocruorins (*cf.* Table IX). In some species the particle weight appears to be as low as 219,100; in others, as high as 2,850,000.

Pigmented Plant Proteins.—Certain sea algae contain the chromoproteins *phycoerythrin* and *phycocyan*. These have been studied by a number of workers. Kylin [55] prepared them in crystalline form, identified them as of protein nature, studied their behavior toward various reagents, including enzymes, acids, and alkalies, and their reactions toward heat and light.

Later Kitasato [56] isolated a reddish-violet, water-soluble pigment to which he gives the formula $C_{48}H_{120}O_{18}N_{12}$ from phycoerythrin. This is further hydrolyzed by acids to yield a blue pigment, insoluble in water, soluble in organic solvents, and having a composition, $C_{30}H_{54}O_{12}N_6 \cdot 1.5\ H_2O$. He notes that phycocyan yields, on peptic digestion, the final blue pigment directly, the intermediate compound not being obtained.

Svedberg (Table IX) studied the light absorption of these proteins and their minimal particle weight as determined in the ultracentrifuge. The particle weight ranged from 106,000 to 208,000, depending upon the biological source of the protein. The chemical nature of the colored group still remains for future investigation.

Melanoproteins.—Black wool by treatment with extremely dilute

[54] Kobayashi, S., *Science Repts. Tohoku Imp. Univ., 4th Series*, 8: 277–285 (1933).

[55] Kylin, H., Über Phykoerythrin und Phykocyan bei Ceramium rubrum (Huds.) Ag., *Z. physiol. Chem.*, 69: 169–239 (1910).

[56] Kitasato, Z., Biochemische Studien über Phycoerythrin und Phycocyan, *Acta Phytochimica*, 2: 75–98 (1926).

alkalies [57] yields a material of protein nature which is intensely black but which on acid hydrolysis yields about 90 per cent of its weight in amino acids. This material is apparently a conjugated protein in which melanin is the associated chromogen. Inasmuch as no similar compound could be isolated from non-pigmented wool, and inasmuch as certain other pigmented structures, such as human hair, black rabbit fur, and black feathers, did not yield a similar product, it seems reasonable to believe that the pigment of black wool exists in the form of *melanoproteins* rather than in the form of melanin which is not associated with the protein molecule. Brown horse hair and brown cattle hair yield similar products. The protein, as isolated, appears to be relatively unaltered, inasmuch as it still will undergo heat coagulation after it has been separated and purified by dialysis.

The Yellow Oxidation Enzyme.—Warburg and Christian [58] prepared an oxidation enzyme which apparently controls cell respiration in the absence of iron. This was later shown [59] to be a "flavin-protein," *i.e.*, a conjugated protein containing flavin esterified with phosphoric acid, the phosphoric acid in turn providing the link of the flavin phosphate with the protein. The flavin appears to be identical with riboflavin (ovoflavin or lactoflavin) or vitamin G (*vide infra*). The chromoprotein has an isoelectric point at *p*H 5.25 and contains 15.9 per cent nitrogen. Theorell split off the flavin phosphate and found that it had no enzyme activity and the residual protein likewise was without activity. The two fractions were then recombined, and the original activity was regained. Apparently there is a stoichiometric combination between the flavin phosphate and the protein, since the activity of the flavin-phosphate-protein combination increased in direct proportion to the amount of flavin phosphate added up to a maximum, after which further additions of flavin phosphate had no effect in altering the enzyme activity.

The Carotenoid Proteins.—Visual purple of the retina of the eye appears to be a chromoprotein in which the prosthetic group is a carotenoid, retinene. Retinene is closely related to vitamin A, inasmuch as in the absence of vitamin A the normal amount of visual purple fails to develop and a nutritional type of "night blindness" [60] ensues.

[57] Gortner, R. A., Studies on Melanin, I. Methods of Isolation, The Effect of Alkali on Melanin, *J. Biol. Chem.*, 8: 341–363 (1910); On Melanin, *Biochem. Bull.*, 1: 207–215 (1911).

[58] Warburg, O., and Christian, W., Über ein neues Oxydationsferment und sein Absorptionsspektrum, *Biochem. Z.*, 254: 438–458 (1932); Über das gelbe Oxydationsferment, *ibid.*, 257: 492 (1933).

[59] Theorell, H., Das gelbe Oxydationsferment, *Biochem. Z.*, 278: 263–290 (1935).

[60] The nutritional night blindness associated with the lack of vitamin A should not be confused with the long-known and genetically interesting hereditary night blindness. In the hereditary night blindness we have one of the striking examples of a sex-linked character. The affliction is transmitted through the females, who

Visual purple, when acted upon by light, goes to visual yellow. Visual yellow, when heated, dissociates into a protein and vitamin A.[61] The particle weight[62] of visual purple computed from the apparent diffusion coefficient is in the neighborhood of 810,000.

IX. THE GLYCOPROTEINS.—We have already noted that egg albumin contains a carbohydrate radical. The amount of carbohydrate which is present, however, is extremely small in comparison with the amount of carbohydrate in the group known as the mucins and the mucoids. The mucin contained in the submaxillary glands has been reported to contain 23.5 per cent, salivary mucin to contain as high as 36.9 per cent, and tracheal mucin from 34 to 37 per cent, of carbohydrate. The group which is associated with the protein has been studied particularly by Levene[63] and his co-workers. He distinguishes two types of groups associated with the protein, *i.e.*, chondroitin sulfuric acid and mucoitin sulfuric acid.

Chondroitin sulfuric acid on hydrolysis yields a reducing disaccharide, *chondrosin*, composed of 1 molecule of chondrosamine (aminogalactose) combined with a molecule of glucuronic acid. Both the amino group and the carboxyl group are free, the linkage apparently being from the carbonyl group of the chondrosamine to the fourth carbon of the glucuronic acid. Other hydrolytic products are glucuronic acid, acetic acid, and sulfuric acid.

Chondroitin sulfuric acid contains 2 chondrosamine residues in which the amino group is acetylated, and the hydroxyl group on the sixth carbon atom is esterified with sulfuric acid. These acetylated and esterified chondrosamine residues are linked through glycosidal linkages to a disaccharide acid composed of 2 molecules of glucuronic acid as shown in the following graphic formula. Provisionally the linkage from the chondrosamine residue is to the fourth carbon of the glucuronic acid.

The decomposition products of *mucoitin sulfuric acid* are mucosin, a disaccharide composed of glucuronic acid, and glucosamine. *Mucosin* apparently has a structure very similar to chondrosin, excepting that the amino sugar is derived from glucose instead of galactose.

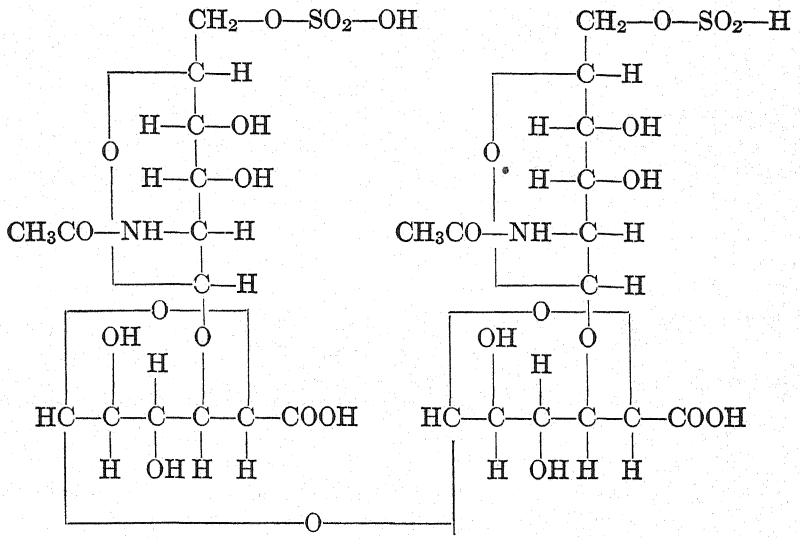
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do not show night blindness but whose sons are night blind. The daughters of these sons are normal but transmit the affliction to their sons. Thus, the affliction is evidenced in the male line but transmitted by the female line. The hereditary form is not due to the absence of vitamin A, and vitamin A therapy is of no avail, since the rods of the retina are lacking.

[61] Mirsky, A. E., The Visual Cycle and Protein Denaturation, *Proc. Natl. Acad. Sci. U. S.*, 22: 147–149 (1936); *cf.* also Wald, G., Carotenoids and the Visual Cycle, *J. Gen. Physiol.*, 19: 351–368 (1935).

[62] Hecht, S., Chase, A. M., and Shlaer, S., The Diffusion Coefficient and Molecular Size of Visual Purple, *Science*, 85: 567–568 (1937).

[63] Levene, P. A., Hexosamines, Their Derivatives, and Mucins and Mucoids, The Rockefeller Institute for Medical Research Monograph 18, 104 pp. (1922).



Chondroitin sulfuric acid

These amino sugar residues likewise occur in the acetylated form and are linked with a diglucuronic acid residue through glycosidal linkages. The sulfuric acid ester residue is much less stable in mucoitin sulfuric acid, so presumably the esterification is on a different hydroxyl group from that in chondroitin sulfuric acid.

When the sulfuric acid radical is removed from the chondroitin sulfuric acid or from the mucoitin sulfuric acid, substances are formed which are non-reducing, relatively inert chemically, and in many respects resemble gum acacia (gum arabic) in properties. These compounds are highly hydrophilic and probably account for the very hydrophilic properties of the mucins and mucoids with which they are associated in the conjugated proteins.

Chondroitin sulfuric acid has found a use in medicine in the treatment of migraine. Alvarez [64] cites Crandall and Roberts as having observed that relief was afforded in about 50 per cent of the cases where chondroitin sulfuric acid was administered.

X. The Phosphoproteins.—*Casein* of milk and *vitellin* of egg yolk are the two most important phosphoproteins. Casein, because of its ease of preparation, has been most extensively investigated. The chemistry of casein, including its uses in industry and the arts has been adequately discussed by Sutermeister and co-authors,[65] and in the

[64] Alvarez, W. C., The Present Day Treatment of Migraine, *Proc. Staff Meetings, Mayo Clinic*, 9: 22–27 (1934).

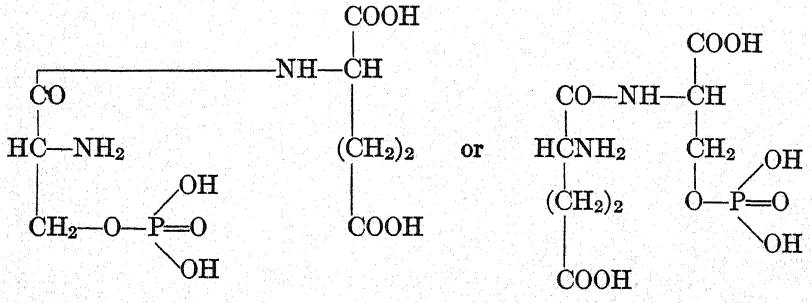
[65] Sutermeister, Edwin, Casein and Its Industrial Applications, Chemical Catalog Company, New York (1927). Note in particular Chapter I.

American Chemical Society Monograph dealing with dairy science.[66]

Casein is the characteristic protein of the milk of all species of mammals, even the egg-laying monotreme or spiny anteater, *Echidna aculeata multiaculeata*, secreting in its mammary glands a protein having the characteristics of casein.[67] Similarly, vitellin appears to be the characteristic protein of egg yolk, although the various vitellins have not been investigated as extensively as have the caseins.

The caseins are acted upon by a special enzyme, rennin, which converts them into another protein, *paracasein*, having somewhat different properties. Whether rennin actually brings about a hydrolytic cleavage resulting in a paracasein with a smaller micellar weight than the original casein or whether we are dealing with a special type of denaturation is still uncertain.

The prosthetic group of the phosphoproteins is *ortho*-phosphoric acid esterified to the hydroxyl group of hydroxyamino acids. Apparently Posternak [68] was the first one to suggest such an ester linkage. Almost simultaneously Rimington [69] attacked the same problem and isolated a phosphopeptone containing a large fraction of the organic phosphorus of casein. Later Levene and Hill [70] and Schmidt [71] almost simultaneously but independently reported the isolation of a phosphodipeptide, glutamyl serine phosphate, which Levene notes possesses one or the other of the following alternative formulas:



[66] Associates of Lore A. Rogers, Fundamentals of Dairy Science, Second Edition, American Chemical Society Monograph Series, Reinhold Publishing Corporation, New York (1935).

[67] Marston, H. R., The Milk of the Monotreme—*Echidna aculeata multiaculeata, Australian J. Exptl. Biol. and Med. Sci.*, 3: 217–220 (1926).

[68] Posternak, S., Sur le noyau phosphoré de la caséine, *Compt. rend.*, 184: 306–307 (1927).

[69] Rimington, C., The Phosphorus of Caseinogen. I. Isolation of a Phosphorus-Containing Peptone from Tryptic Digests of Caseinogen. II. Constitution of Phosphopeptone, *Biochem. J.*, 21: 1179–1193 (1927).

[70] Levene, P. A., and Hill, D. W., On a Dipeptide Phosphoric Acid Isolated from Casein, *J. Biol. Chem.*, 101: 711–718 (1933).

[71] Schmidt, G., Über den phosphorsäure haltigen Baustein des Caseinmoleküls, *Proc. 14th Intern. Congress Physiol.*, p. 228 (1933); *Z. physiol. Chem.*, 223: 86–88 (1934).

The fact that phosphoric acid is esterified on the —OH group of serine was proved by the isolation of serine phosphoric acid.[72] Rimington's paper indicates that the phosphoric acid may be esterified both as the monophosphate and as the diphosphate esters. Through the differential action of bone phosphatase, which does not hydrolyze diphosphoric acid esters, and kidney phosphatase, which hydrolyzes both the mono- and diesters, he concludes that the phosphoric acid in casein exists in a ratio of 2 monoesters to 1 diester. If that be true, phosphoric acid may, when in the diester form, act as a bridge between two polypeptide chains and link such chains together in the larger casein molecule.

Casein in comparison with most proteins is a relatively strong acid, and its titration curve with sodium hydroxide shows a sharp inflection between *p*H 6.0 and 7.5 (*cf.* Fig. 43). It is in the same range that the second hydrogen of phosphoric acid is neutralized, so that presumably a very considerable part of the alkali-binding capacity of casein is attributable to the secondary hydrogen of the phosphoric acid. An extensive study of casein has been conducted in Sörensen's laboratories with the conclusion that casein is a reversible dissociable component system,[73] and this point of view is borne out by sedimentation studies in Svedberg's laboratories where the sols were found to be heterodisperse, the particle weight varying from 75,000–100,000 up to 375,000, depending upon the fraction which was studied and the technic employed in the preparation of the protein sols.

XI. THE NUCLEOPROTEINS.—The nucleoproteins consist of a protein group, usually a *histone* or *protamine*, combined with *nucleic acids*. The nucleic acids have been extensively studied, particularly by Jones [74] and Levene.[75]

The nucleic acids which have been studied have been derived mainly from either the thymus gland or from yeast, the former being taken as a representative of the nucleic acids of animal origin, the latter of plant origin. On hydrolysis all nucleic acids yield phosphoric acid, a carbohydrate, two pyrimidine bases, and two purine bases. For a long time the statement was generally accepted that plant nucleic

[72] Lipmann, F., Über die Bindung der Phosphorsäure in Phosphorproteinen I. Isolierung einer phosphorhaltigen Aminosäure (Serinphosphorsäure) aus Casein, *Biochem. Z.*, 262: 3–8 (1933); *cf.* also *ibid.*, 262: 9–13 (1933).

[73] Kondo, K., Studies on Casein, *Compt. rend. trav. lab. Carlsberg*, Vol. XV, No. 8, 40 pp. (1925); Linderström-Lang, K., and Kodama, S., Studies on Casein. I. On the Solubility of Casein in Hydrochloric Acid, *ibid.*, Vol. XVI, No. 1, 62 pp. (1925); Linderström-Lang, K., Studies on Casein. III. On the Fractionation of Casein, *ibid.*, Vol. XVII, No. 9, 116 pp. (1929).

[74] Jones, W., Nucleic Acids, Their Chemical Properties and Physiological Conduct, Longmans, Green and Company, New York (1914).

[75] Levene, P. A., and Bass, L. W., Nucleic Acids, Am. Chem. Soc. Monograph 56, Chemical Catalog Company, New York (1931).

acids were all alike but that they differed from animal nucleic acids which were likewise all alike, the difference being that plant nucleic acids contained a pentose, whereas the carbohydrate of the animal nucleic acids was a hexose, and also that one pyrimidine base, uracil, occurred only in the plant nucleic acids, whereas another, thymine, occurred only in animal nucleic acids. Recent investigations have shown, however, that the above statements are not necessarily true.

Levene identified a pentose, *d*-ribose, in animal nucleic acids, and Jones and Perkins [76] reported that uracil is probably derived from cytosine by secondary reactions in the method of isolation. This, however, is disputed by Levene.[77] Johnson and Coghill,[78] in addition, report a new pyrimidine, 5-methylcytosine, in the nucleic acid of the tubercle bacillus. Johnson earlier [79] reported thymine in the nucleic acid of the tubercle bacillus, so that it would appear that the early distinction between plant and animal nucleic acids is pretty well broken down.

The evidence for a hexose in certain nucleic acids was based not upon the isolation of the sugar itself, but rather upon the formation of levulinic acid (γ-ketovalerianic acid) when the nucleic acid is hydrolyzed. Levulinic acid is a characteristic decomposition product of hexoses. Later work [80] indicates that a hexose is not present in thymonucleic acid but that instead the sugar is *d*-desoxyribose, $CH(OH)—CH_2—CH(OH)—CH(—O—)—CH_2OH$. This sugar gives rise to levulinic acid and accounts for the formation of that acid from those nucleic acids which have been supposed to contain a hexose.

d-Ribose was identified by Levene and Jacobs in a series of papers during the years 1908–1911. Rather interestingly, neither this pentose nor desoxyribose was known to occur in nature until they were discovered as decomposition products of nucleic acid. Fischer, during his studies on the carbohydrates, had synthesized *d*-ribose in 1901 and desoxyribose in 1913. We have, thus, examples of synthetic products prepared first in a chemical laboratory which after a number of years were found to be identical with what are probably the most im-

[76] Jones, W., and Perkins, M. E., The Nitrogenous Groups of Plant Nucleic Acid, *J. Biol. Chem.*, 62: 557–564 (1925).

[77] Levene, P. A., On the Nitrogenous Components of Yeast Nucleic Acid, *J. Biol. Chem.*, 67: 325–327 (1926).

[78] Johnson, T. B., and Coghill, R. D., Researches on Pyrimidines. CIII. The Discovery of 5-Methyl-Cytosine in Tuberculinic Acid, the Nucleic Acid of the Tubercle Bacillus, *J. Am. Chem. Soc.*, 47: 2838–2844 (1925).

[79] Johnson, T. B., and Brown, E. B., The Pyrimidines Contained in Tuberculinic Acid. The Nucleic Acid of Tubercle Bacilli, *J. Biol. Chem.*, 54: 731–737 (1922).

[80] Levene, P. A., and London, E. S., The Structure of Thymonucleic Acid, *J. Biol. Chem.*, 83: 793–802 (1929); Levene, P. A., Mikeska, L. A., and Mori, T., On the Carbohydrate of Thymonucleic Acid, *J. Biol. Chem.*, 85: 785–787 (1930).

portant carbohydrates occurring in nature. An interesting philosophical discussion could be built around this question of this "most important carbohydrate." We derive our energy from glucose. The cell nuclei contain either *d*-ribose or *d*-desoxyribose. Is the engine more important than the fuel which runs it, or is the fuel more important than the engine?

Recently a new sugar containing sulfur has been isolated from yeast, and there is certain evidence that it may be combined in a nucleic acid. This point will be returned to later.

The decomposition products of the nucleic acids as usually obtained may be diagrammed as follows:

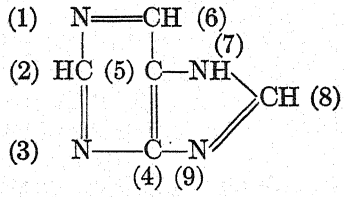
H_3PO_4

Carbohydrates { Pentoses—*d-ribose*, *d-desoxyribose*

Bases { Purines { guanine, adenine } aminopurines; xanthine, hypoxanthine } oxypurines

Bases { Pyrimidines { cytosine, thymine, uracil }

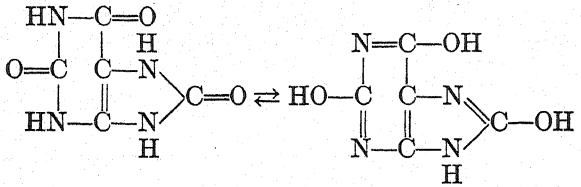
Guanine and adenine are the only naturally occurring purines in the nucleic acids. Xanthine and hypoxanthine are derived from these by the action of acids or enzymes. Purine, the mother substance of the purine bases, does not occur in nature but has been synthesized and has the following structure, the ring being numbered, as shown in the diagram:



The various derivatives with which we are concerned are as follows:

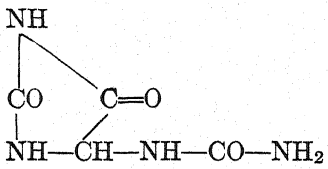
Guanine = 2-amino-6-oxypurine.
Adenine = 6-aminopurine.
Guanine + enzyme (guanase) = 2.6-dioxypurine = *xanthine.*
Adenine + enzyme (adenase) = 6-oxypurine = *hypoxanthine.*
Hypoxanthine by oxidation goes to xanthine.
Xanthine + oxygen + enzyme = *uric acid,* 2.6.8-trioxypurine.

The oxygenated purine or pyrimidine compounds undergo keto ⇌ enol isomerism and accordingly may exist in either the enol form or the keto form, or as an equilibrium mixture of the two forms:



Keto form of uric acid — Enol form of uric acid

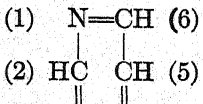
Uric acid is the primary end product of purine metabolism in man and in the anthropoid ape. Monkeys excrete only traces. In the lower animals it is further oxidized to allantoin:



which in some cases is excreted as such, in others is further oxidized to urea in the liver. When the liver is removed, the purines are excreted as uric acid. Allantoin excretion decreases following liver removal (dog).[81] The enzymes adenase and guanase are *deaminases*, the further oxidation to uric acid being produced by *oxidases*.

Besides the purines noted above, some others are important from a phytochemical standpoint. These are *caffeine*, 1.3.7-trimethylxanthine, *theobromine*, 3.7-dimethylxanthine, and 1.3.7.9-tetramethyl-2.6.8-trioxypurine (tetramethyl uric acid), which has recently been found to occur in tea. Theobromine occurs in cocoa beans to the extent of 1.5 to 2.4 per cent and in chocolate to the extent of 1 to 2 per cent. Caffeine occurs in tea, coffee, kola nut, cocoa bean, etc. The coffee bean contains about 1 per cent, tea leaves 1.5 to 2.5 per cent. It is a diuretic and cerebral stimulant and has a pronounced stimulating action on the heart.

The pyrimidine compounds found in nucleic acids are derivatives of the pyrimidine nucleus:



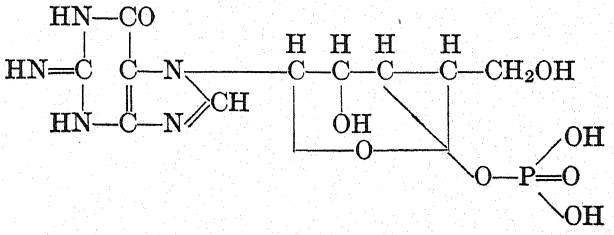
Pyrimidine

[81] Bollman, J. L., and Mann, F. C., Uric Acid Metabolism in the Absence of the Liver, *Proc. Staff Meeting, Mayo Clinic*, 7: 631–632 (1932).

The only important derivatives from our standpoint are:

cytosine, 2-oxy-6-aminopyrimidine;
thymine, 2.6-dioxy-5-methylpyrimidine;
uracil, 2.6-dioxypyrimidine;
5 *methylcytosine,* 2-oxy-5-methyl-6-aminopyrimidine.

The structure of nucleic acids has been largely arrived at by a study of the *mononucleotides, i.e.*, compounds containing a single molecule each of phosphoric acid, a carbohydrate, a purine, or a pyrimidine base. Typical mononucleotides are guanylic acid and inosinic acid. These decompose respectively into phosphoric acid, guanine, and *d*-ribose, and phosphoric acid, hypoxanthine, and *d*-ribose. The structure of guanylic acid is probably as follows:



Guanylic acid (7-guanine-3-phosphoribofuranoside)

where the union between the carbohydrate and the heterocyclic ring is directly from carbon to nitrogen. The sugar in the adenose nucleotide and probably in the other nucleotides has the furanose or γ-oxide structure, and the phosphoric acid is linked to either the third carbon or the fifth carbon atom of the sugar. Dubos [82] recently described a rather unusual enzyme, a polyneucleotidase which hydrolyzes nucleic acid to nucleotides. It was prepared from polymorphonucleoleucocytes, liver, pancreas, spleen, or lungs, and has an optimum activity at about *p*H 4–5, although it retains most of its activity even up to *p*H 1 or *p*H 7.5. It is extremely resistant toward heat, practically all the activity remaining after 5 minutes in a boiling-water bath. Its activity increases up to 75°, then decreases sharply so that there is no action at 85° C., but if the preparation which has been held at 85–90° is cooled to 60°, rapid enzymatic hydrolysis of nucleic acid ensues. This polynucleotidase attacks yeast nucleic acid but not thymus nucleic acid. The enzyme is resistant to trypsin but is readily destroyed by pepsin. Dubos suggests that it appears to be a protein.

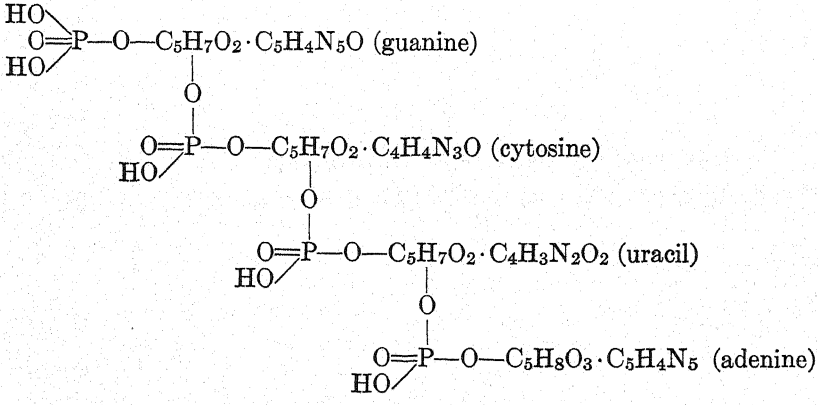
Muscle contains compounds very similar to the nucleic acid nucleotides. The adenine-carbohydrate-phosphoric acid compound of

[82] Dubos, R. J., The Decomposition of Yeast Nucleic Acid by a Heat Resistant Enzyme, *Science*, 85: 549–550 (1937).

muscle [83] contains three molecules of phosphoric acid. Two of these are readily removable by hydrolysis; one is resistant to hydrolysis. The two which are readily removable are *ortho*-phosphoric acid. The resistant one is *pyro*-phosphoric acid.

The nucleotides may be converted by the proper reagents into *nucleosides* with the loss of phosphoric acid, a nucleoside being defined as the compound in which a purine or a pyrimidine is joined to a carbohydrate. Various nucleosides have been isolated. Thus, guanosine is a compound composed of one molecule each of guanine and *d*-ribose. Adenosine is composed of a molecule each of adenine and *d*-ribose, etc.

Nucleotides then may be considered as compounds of nucleosides with phosphoric acid, and apparently four nucleotides unite to make a molecule of nucleic acid. Levene and Jacobs, in hydrolyzing yeast nucleic acid, obtained phosphoric acid and four nucleotides containing guanine, adenine, cytosine, and uracil, respectively. These four nucleotides were apparently condensed with the loss of three molecules of water to form yeast nucleic acid. The union between the nucleotides is between the carbohydrate groups and phosphoric acid. Levene's formula for a hypothetical nucleic acid is as follows:

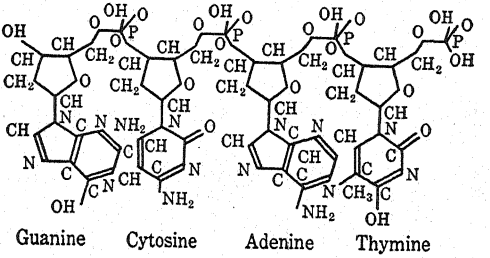


Whether the nucleic acids exist in the open-chain structure or in the closed-chain structure is still uncertain. Figure 133 shows two possibilities.

We are still uncertain with regard to the positions of the various nucleotides in the nucleic acid molecule. Accordingly different ar-

[83] Fiske, C. H., and Subbarow, Y., Phosphorus Compounds of Muscle and Liver, *Science*, 70: 381–382 (1929); Embden, G., and Zimmermann, M., Über die Bedeutung der Adenylsäure für die Muskelfunktion, *Z. physiol. Chem.*, 167: 137–140 (1927); Embden, G., and Schmidt, G., Über Muskeladenylsäure und Hefeadenylsäure, *Z. physiol. Chem.*, 181: 130–139 (1929).

rangements of the nucleotides may give rise to different stereoisomeric nucleic acids. The purines appear to be linked to the sugars through a glycosidal linkage at position-7 on the purine ring, and the pyrimidines appear to be similarly linked through position-3. All the sugar residues appear to have the γ-oxide structure, and those purine nucleotides derived from thymonucleic acid have the phosphoric acid esteri-



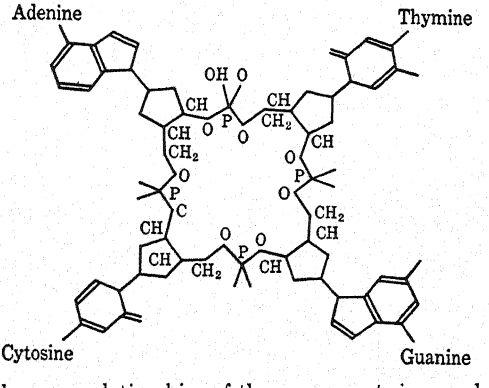


FIG. 133.—Possible space relationships of the components in a molecule of a nucleic acid. (After Wrinch.)

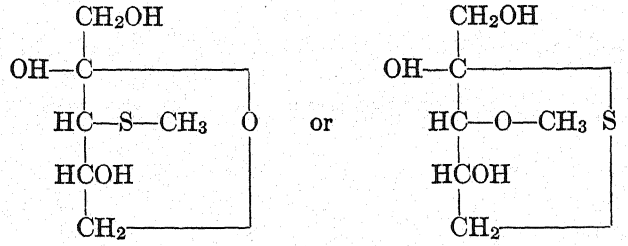
fied on the fifth carbon atom of the ribose, whereas in yeast nucleic acid the phosphoric acid is esterified on the third carbon atom. The pyrimidine ribonucleotides appear to be esterified with phosphoric acid on the third carbon atom of the sugar.

Mandel and Dunham [84] isolated an adenine nucleoside from yeast which apparently had the formula $C_{11}H_{15}N_5O_5$, but which could not be identified with any known nucleoside. Levene [85] later isolated the

[84] Mandel, J. A., and Dunham, E. K., Preliminary Note on a Purine-Hexose Compound, *J. Biol. Chem.*, 11: 85–86 (1912).

[85] Levene, P. A., Adenosin Hexoside from Yeast, *J. Biol. Chem.*, 59: 465–472 (1924).

same compound and reported that the sugar differed from all known ketohexoses. Later, Suzuki, Odake, and Mori [86] isolated an adenine-carbohydrate compound from yeast *and found that the sugar contained sulfur*. They believed it to be a methylthiopentose. Levene and Sobotka [87] then reinvestigated the nucleoside which Levene had previously isolated, and found that the sugar was not a ketohexose but that it contained sulfur and was identical with the product isolated by Suzuki, Odake, and Mori. Levene suggests that it has one of the following alternative formulas:



i.e., it is a methylated thiopentose. The $—OCH_3$ or the $—SCH_3$ may be on the fourth instead of the third carbon. This compound is the only naturally occurring sulfur-containing carbohydrate which has been reported in the literature. Whether or not this sugar occurs in nucleic acids is still uncertain. Future workers should, however, make tests for the presence of sulfur when they are studying nucleic acids or decomposition products of nucleic acids.

Rose [88] presents in his paper on purine metabolism an excellent review of the changes which take place in the various purine and pyrimidine derivatives under the influence of various reagents and enzymes, and also discusses the fate of the various purine derivatives in man and in animals. The question as to whether or not animals are able to synthesize the purine derivatives needed for nucleic acid formation has not been satisfactorily answered.

Robertson,[89] as the result of feeding experiments (mice), came to the conclusion that nucleic acid decreased in old tissues and that the

[86] Suzuki, U., Odake, S., and Mori, T., Über einen neuen schwefelhaltigen Bestandteil der Hefe, *Biochem. Z.*, 154: 278–289 (1924).

[87] Levene, P. A., and Sobotka, H., The Thio-Sugar from Yeast, *J. Biol. Chem.*, 65: 551–554 (1925).

[88] Rose, W. C., Purine Metabolism, *Physiol. Rev.*, 3: 544–602 (1923).

[89] Robertson, T. B., The Duration of Life, *Rept. Australian Assoc. Advancement Science*, 16: 863–882 (1926); On the Influence of Nucleic Acids of Various Origin upon the Growth and Longevity of the White Mouse, *Australian J. Exptl. Biol. Med. Sci.*, 5: 47–67 (1928); Robertson, T. B., and Dawbarn, M. C., Preliminary Communication on the Influence of Age of the Animal upon the Nucleic Acid and Coagulable Nitrogen Content of the Tissues of Sheep, *Australian J. Exptl. Biol. Med. Sci.*, 6: 261–273 (1929).

decrease in nucleic acid was associated with senescence. In those cases where he fed nucleic acid he reports that the duration of life was very appreciably lengthened. If Robertson's results are substantiated, this would appear to indicate that the synthetic mechanism in animals may not provide optimum amounts of nucleic acid.

T. B. Johnson states that, since purines can be readily synthesized from pyrimidines, it seems extremely probable that pyrimidines are the precursors of the purines in plants. However, cytosine and uracil have never been found in the free condition in plants but are always combined as nucleosides. On the other hand, purines occur free, probably as an end product of nitrogen metabolism, for example, theobromine and caffeine in plants, and uric acid in man.

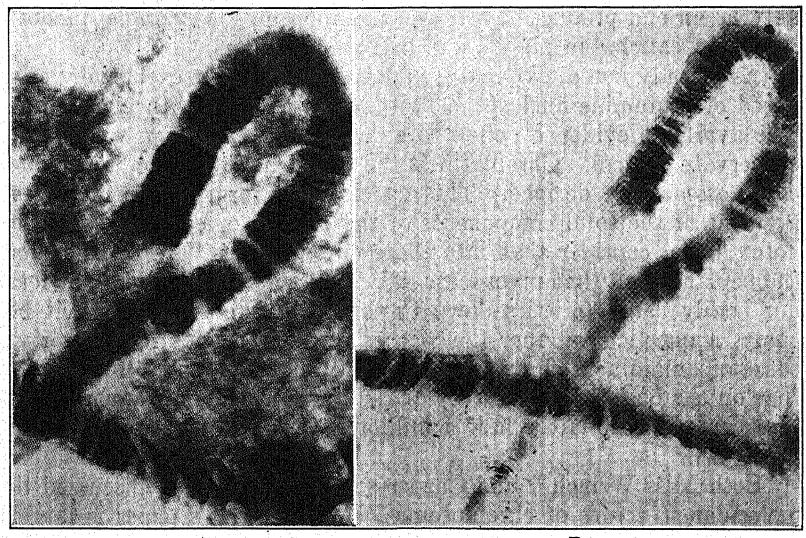
As already noted, *chromatin* appears to be almost exclusively composed of protamine nucleate, a protamine combined with nucleic acid. The available evidence points to either a true salt formation or to a coacervate system. Chromatin is the chemical substance from which *chromosomes* are derived. The nucleic acids form approximately 40 per cent of the solid components of the chromosomes, and as Leathes notes, if we consider that into these chromosomes "are packed from the beginning all that preordains, if not our fate and fortunes, at least our bodily characteristics down to the color of our eyelashes, it becomes a question whether the virtues of nucleic acids may not rival those of amino acid chains in their vital importance." Leathes notes that on the basis of 40 per cent of the nucleic acids in the chromatin, there are approximately half a million molecules of nucleic acid in a sperm cell.

Both Miss Wrinch [90] and Caspersson [91] have recently discussed the molecular structure of chromosomes. Miss Wrinch's paper is largely a theoretical discussion of the available literature. Caspersson's paper, on the other hand, is an extensive experimental study. Both reach essentially the same conclusions. Caspersson studied the structure of the chromosomes by making use of the ultraviolet absorption spectra. Nucleic acid has a great absorption in the ultraviolet. Proteins likewise have marked absorption in the same region, but Caspersson was able to digest out the proteins, leaving what he believes to be the nucleic acid residues, and thus to demonstrate that nucleic acid is deposited in the chromosomes in bands which correspond to the bands which the cytologist has observed in many of the chromosomes and which appear to be associated with the genes of the geneticist. Figure 134*A* shows parts of two of the giant salivary gland chromosomes of *Chironomus* larvae as photographed by Caspersson, showing dark ultraviolet

[90] Wrinch, D. M., *Protoplasma, loc. cit.*

[91] Caspersson, T., Über den chemischen Aufbau der Strukturen des Zellkernes, *Skandinavisches Arch. für Physiologie*, 73 (Supplement No. 8): 151 pp., 107 Figs., (1936).

absorption bands. Figure 134*B* shows the same preparation after the protein had been digested out in a 24-hour proteolytic digestion. The dark bands in Fig. 134*B* occupy essentially the same positions as in Fig. 134*A*, but the absorbing protein material between the areas where the nucleic acid is concentrated has been digested out so that Caspersson believes that the bands shown in Fig. 134*B* demonstrate the localization of nucleic acid within the chromosome structure. Miss Wrinch postulates the structure of chromosomes as being made up of a protein (protamine) warp and a nucleic acid woof. The nucleic acid



A B

FIG. 134.—(*A*) Chromosomes from salivary glands of *Chironomus* larvae photographed with ultraviolet microscope technic. The dark bands are due to an intense absorption of ultraviolet light by protamine-nucleate. (*B*) Same preparation as (*A*) after the protein portion of the chromosomes had been solubilized by digestion with proteolytic enzymes for 24 hours. Note that nucleic acid is unaffected by proteolytic enzymes and remains in a banded structure. (After Caspersson.)

is linked to the *basic* groups of the protamine but is not attached to the monoaminomonocarboxylic acid residues. There would thus be a banding of nucleic acid surrounding the parallel chains of protamines in those areas where the dibasic amino acids are concentrated. This would account for the banded structure of the chromosomes. The coacervate viewpoint would lead to the same picture. The negatively charged anions of the nucleic acid would be attracted to the positively charged areas of the protamine so that localized concentrations of nucleic acid would occur along the protamine structure. The only

difference between this picture and a true salt formation would be that in the coacervate the water shells would prevent the positively charged hydrated cations from completely neutralizing the hydrated negatively charged anionic residues. Thus, the positive and negative charges would still retain their identities, the structure being held together by electrostatic attractions but the charged areas being held apart by the repulsion of the oriented water shells.

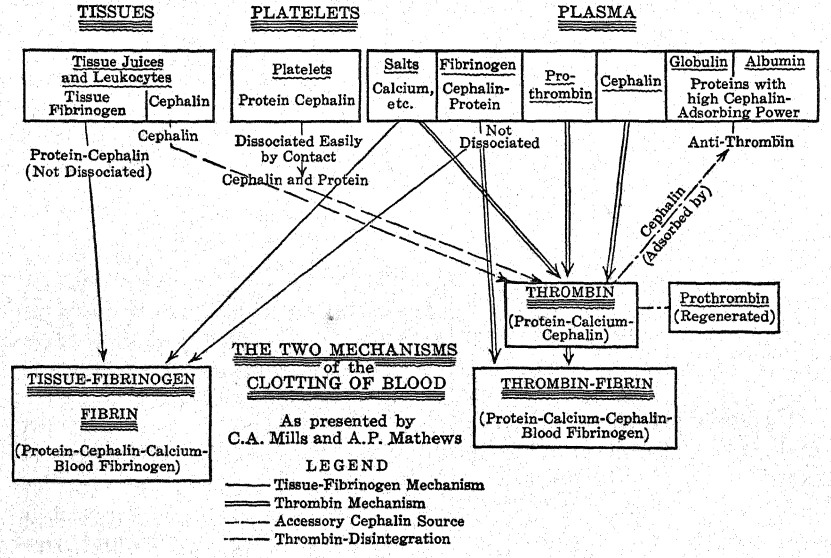
XI. THE LECITHOPROTEINS.—The literature is somewhat confused in regard to this class of proteins, some workers feeling that the lecithoproteins are definite compounds, others that they represent mixtures of proteins with lecithin or other phospholipids in varying proportions. Within recent years, however, there appears to be a rather general trend toward viewing the combinations between protein and lipids which occur in certain cells and tissues as representing a conjugated protein in spite of the fact that the lipid fraction can be very largely removed by extraction with fat solvents. Such a viewpoint would probably lead to including the vitellin of egg yolk in the group of lecithoproteins, since it occurs together with large amounts of lecithin in the egg yolk and cannot be completely freed from lecithin except by rather drastic extraction.

Perhaps the best example of a lecithoprotein which has been studied extensively is *tissue fibrinogen.*[92] Mills finds that the tissues contain a substance which accelerates the clotting of blood and that this substance is composed of a protein and a phospholipid in the ratio of approximately 58.4 per cent protein and 41.6 per cent of a phospholipid. A large part of the phospholipid can be removed by extraction with fat solvents. A part, however, is very firmly held and is not completely removed except by methods which tend to partially hydrolyze the protein. The purified material possesses extraordinary power in causing blood clotting. Lung tissue is high in the so-called tissue fibrinogen. If the phospholipid is removed from the lipoprotein by fat solvents, neither the protein fraction nor the phospholipid fraction is capable of accelerating blood clotting. If, however, the two extracts are recombined, a large part of the original activity is re-

[92] Mills, C. A., The Activity of Lung Extract, as Compared to Extracts of Other Tissues, in Inducing Coagulation of the Blood, *J. Biol. Chem.*, 40: 425–433 (1919); Chemical Nature of Tissue Coagulins, *ibid.*, 46: 135–165 (1921); The Action of Tissue Extracts in the Coagulation of Blood, *ibid.*, 46: 167–192 (1921); Mills, C. A., Raap, G., and Jackson, D. E., A Note on the Relation between the Blood-Coagulating and the Smooth Muscle-Contracting Properties of Tissue Extracts, *J. Lab. Clin. Med.*, 6: 374–388 (1921); Mills, C. A., and Guest, G. M., The Role of Tissue Fibrinogen (Thrombokinase) in Fibrin Formation and Normal Clotting, *Am. J. Physiol.*, 57: 395–419 (1921); Mills, C. A., Blood Clotting Studies in Hemophilia, *ibid.*, 76: 632–641 (1926); The Manner of Action of the Protein Fraction of Tissue Fibrinogen as a Blood Anticoagulant, *ibid.*, 76: 651–659 (1926); Considerations of the Problem of Blood Clotting, *Am. J. Med. Sci.*, 172: 501–510 (1926).

gained. Tissue fibrinogen is such a powerful blood coagulant as to cause the cessation of hemorrhage even in cases of hemophilia, and has been prepared for either subcutaneous injection or for taking by mouth. If injected intravenously, death ensues, due to the formation of clots in the blood vessels. Mills believes that the presence of such materials in the tissues is the protective agency which the body provides against excessive hemorrhage.

Mills and Mathews have studied the mechanism of the clotting of blood and find that both in the normal clotting process and the clotting process induced by tissue fibrinogen, there is the interaction of both protein and phospholipid. The following chart prepared by Mills and Mathews indicates the two mechanisms of blood clotting.



Troensegaard and Koudahl [93] report the presence of cholesterol as a group associated with the globulin of blood serum.

It seems probable that in many instances the proteins existing in the tissues may be associated with either fats, lipids, or fatty acids. However, in our method of purification of the protein we remove these groups by fat solvents. This is perhaps simply a restatement of the remarks already credited to Abderhalden where he notes that the proteins prepared and purified for chemical study may in many instances bear little resemblance in their physical properties to the proteins as they actually exist in the cells and tissues.

[93] Troensegaard, N., and Koudahl, B., Cholesterin als prostethische Gruppe im Serum-Globulin, *Z. physiol. Chem.*, 153: 111–118; 157: 62–63 (1926).

XII. Derived Proteins.—Derived proteins, as already noted, are proteins which have been chemically altered by manipulation subsequent to their isolation. They include such groups as the coagulated proteins, the halogenated proteins, the nitrated proteins, the formaldehyde proteins, deaminized proteins, racemized proteins, and proteans, etc. The literature in this field is so voluminous that mention can be made of only a few of the reactions involved.

Coagulated Proteins.—Perhaps the most studied of the coagulated proteins is the heat-coagulated egg white. When a concentrated solution of egg white is heated, the proteins coagulate and the entire mass sets to a more or less rigid gel. If, however, the egg white has previously been diluted, gel formation does not occur, the protein separating instead in the form of flocs.

We have already noted that coagulation occurs at the interface between an egg albumin solution and air. In this case, surface energy brings about coagulation in the same way that heat energy causes heat coagulation of the albumin. Various other forms of energy likewise will induce coagulation, *e.g.,* irradiating with ultraviolet light,[94] with the rays of a radium salt,[95] or by high pressures,[96] as well as by the application of heat.[97] In each instance energy is added to the system.

Although the coagulation of proteins has been the subject of numerous researches, there is still considerable uncertainty as to exactly what chemical changes occur in the protein molecule. There seems to be almost a unanimity of opinion that heat coagulation as well as surface coagulation is accompanied by a partial dehydration of the micelle

[94] Clark, J. H., The Action of Ultraviolet Light on Egg Albumin in Relation to the Isoelectric Point, *Am. J. Physiol.,* 61: 72–79 (1922); Studies on Radiated Proteins. I. Coagulation of Egg Albumin by Ultraviolet Light and Heat, *ibid.,* 73: 649–660 (1925); *cf.* also Bovie, W. T., A Preliminary Note on the Coagulation of Proteins by Ultraviolet Light, *Science,* 37: 24–25 (1913); The Temperature Coefficient of the Coagulation Caused by Ultraviolet Light, *ibid.,* 37: 373–375 (1913).

[95] Fernau, A., and Pauli, W., Über die Einwirkung der durchdringenden Radiumstrahlung auf anorganische und Biokolloide, I., *Biochem. Z.,* 70: 426–441 (1915).

[96] Bridgman, P. W., The Coagulation of Albumen by Pressure, *J. Biol. Chem.,* 19: 511–512 (1914).

[97] Sörensen, M., and Sörensen, S. P. L., Studies on Proteins VII. On the Coagulation of Proteins by Heating, *Compt. rend. trav. lab. Carlsberg,* Vol. XV, No. 9, 26 pp., (1925); Pauli, Wo., and Weiss, R., Über Beziehungen zwischen kolloiden und konstitutiven Änderungen einiger Proteine. I., *Biochem. Z.,* 233: 381–443 (1931); Pauli, Wo., and Weissbrod, J., Über Beziehungen zwischen kolloiden und konstitutiven Änderungen einiger Proteine, IV. Hitzegerinnung und kolloider Aufbau der Albumine, *Kolloid-Beihefte,* 42: 429–462 (1935); Neurath, H., and Bull, H. B., The Denaturation and Hydration of Proteins. I, *J. Biol. Chem.,* 115: 519–528 (1936); *cf.* also Pauli, Wo., and Valkó, E., Kolloidchemie der Eiweisskörper, 353 pp., Theodor Steinkopff, Dresden and Leipzig (1933).

which could be interpreted as indicating the neutralization of some of the highly polar groups within the molecule, thus lessening the possibility for water-binding. In both heat denaturation and surface denaturation there is a slight but definite shift in the hydrogen-ion concentration of the system, the direction and degree of this shift being determined by the initial *p*H of the egg-albumin sol. The form of the curves for surface denaturation [98] are remarkably similar to those for heat denaturation.[99] These shifts in hydrogen-ion concentration again indicate an alteration in the effective acidic and basic groups of the protein. It seems probable that in some way the zwitterion structure is modified and perhaps in part destroyed. That still deeper-seated changes take place is evidenced by the observation of Mirksy and Anson [100] that tests for the —SH group become positive following denaturation, whereas they are not detectable in the undenatured protein. Neurath [101] suggests that surface denaturation is the unrolling of the globular protein molecule into a surface film, and that, when the globular protein has thus spread, a film structure is developed which is insoluble. It seems reasonable to suppose that an increase in the kinetic energy of the molecule by heating might bring about a similar unfolding of a globular molecule.

The coagulation process occurs in at least two stages, a preliminary stage of *sensitization* followed by a secondary stage in which *flocculation* takes place. This appears to be true irrespective of the means by which coagulation is brought about. Thus, it is possible to prepare an albumin sol which is salt-free, which can be irradiated with ultraviolet light, or heated above the heat-coagulating temperature without the flocking of the protein. Protein sols which have been so treated are in a highly sensitive condition, and flocs are immediately produced when traces of electrolytes are added to such systems. There would seem to be a striking similarity between such systems and a lyophilic → lyophobic sol transformation. The lyophilic sols are not sensitive to the action of traces of electrolytes, whereas lyophobic sols are exceedingly sensitive. Unaltered protein sols are not sensitive to the action of electrolytes, whereas protein sols which have been sensitized by either heat or irradiation become very sensitive to the action of electrolytes.

[98] Bull, H. B., and Neurath, H., the Denaturation and Hydration of Proteins II. Surface Denaturation of Egg Albumin, *J. Biol. Chem.*, 118: 163–175 (1937).

[99] Pauli, Wo., and Kölbl, W., Über Beziehungen zwischen kolloiden und konstitutiven Änderungen einiger Proteine II. Die Wärmedenaturierung der Albumine ausserhalb des isoelektrischen Bereiches, *Kolloid-Beihefte*, 41: 417–460 (1935).

[100] Mirsky, A. E., and Anson, M. L., Sulfhydryl and Disulfide Groups of Proteins III. Sulfhydryl groups of Native Proteins—Hemoglobin and the Proteins of the Crystalline Lens, *J. Gen. Physiol.*, 19: 439–450 (1936).

[101] Neurath, H., The Influence of Denaturation on the Spreading of Proteins on a Water Surface, *J. Phys. Chem.*, 40: 361–368 (1936).

Coagulation can be produced by exposure to ultraviolet light even at 0°. Bovie points out that the coagulation by ultraviolet light has a negligible temperature coefficient, the rate of coagulation being nearly as great at 0° as at higher temperatures. On the other hand, if an albumin solution is rayed for a time only partially long enough to induce coagulation, it is found that the temperature at which it will heat-coagulate has been markedly decreased. It is thus possible to ray an egg albumin solution at 0° for a few minutes and have it remain in an apparently unchanged condition as long as it is kept at 0°. If, however, the temperature is allowed to rise to room temperature, it spontaneously flocculates, or if sufficiently concentrated, sets to a gel.

Miss Clark[102] has made a careful study of the ultraviolet denaturation of egg albumin and concludes that three steps are involved. The first step is a physical process producing a permanent change in the protein molecule which she calls "light denaturation." This step is independent of temperature, occurs over a wide *p*H range, and can be induced in the absence of water. The second step is a chemical reaction between the light-denatured protein molecule and water. It has a high temperature coefficient ($Q_{10} = \pm 10$). She suggests that this may be the first stage in heat denaturation but occurs at a lower temperature when the protein has first been light denatured. The third step is the flocculation.

Although a number of authors have claimed that, if coagulation had proceeded only to the point of incipient turbidity, the reaction could be reversed by cooling the solution or removing it from the source of radiation and allowing it to stand for a time, Adolf,[103] so far as the author is aware, is the first person to claim a successful reversal of the complete heat-coagulation process. She studied serum albumin purified by electrodialysis and found that the hydrogen-ion concentration of the sol decreased from 5.3×10^{-6} to 2.84–2.71×10^{-7}, and the specific electrical conductivity increased from 5.46×10^{-6} to 7.33×10^{-6}, on heat coagulation. By dissolving the coagulated protein in dilute sodium hydroxide and subsequently removing the alkali by electrodialysis, she regenerated a sol having all the properties of the initial serum albumin sol, including hydrogen-ion concentration, specific electrical conductivity, optical rotation, temperature of coagulation, and protective value toward gold and mastic sols. Similar reversals[104] of coagulated proteins have been secured in the case of

[102] Clark, Janet H., The Denaturation of Egg Albumin by Ultraviolet Radiation, *J. Gen. Physiol.*, 19: 199–210 (1935).

[103] Adolf, Mona Spiegel, Hitzeveränderungen des Albumins, *Biochem. Z.*, 170: 126–172 (1926).

[104] Anson, M. L., and Mirsky, A. E., Protein Coagulation and Its Reversal, *J. Gen. Physiol.*, 14: 597–604; 605–609; 725–732 (1931).

hemoglobin, globin, and serum albumin. The reversal in egg albumin has not as yet been effected.

Halogenated Proteins.—When the halogens, chlorine, bromine, or iodine, are introduced into the protein, the question always arises as to whether or not the method of halogenation involves a partial hydrolysis of the protein. In most instances, this is undoubtedly the case. In general, substitution takes place on the aromatic nuclei, notably the benzene rings of tryptophane, tyrosine, and to some extent that of phenylalanine. The same may be said for the nitrated proteins. The Millon and the Adamkiewicz reactions, as a rule, are negative, the characteristic hydrolytic products being the halogenated or nitrated aromatic radicals, particularly that of tyrosine, although the nitrated proteins yield nitroarginine.

Formaldehyde Proteins.—Proteins unite with formaldehyde to form compounds having very different properties from the original protein. Thus, egg albumin treated with formaldehyde is no longer coagulable by heat. Most of the work on the formaldehyde proteins has been carried out using casein, and the manufacture of artificial ivory, galalith, or casein plastics, involving the union of formaldehyde and casein, has resulted in the building up of a large chemical industry, the more or less chemically reactive casein being converted into a relatively inert horn-like mass. A discussion of this process has been given by Brother.[105] Little is known in regard to the chemical reactions which are involved. Yeast and yeast residues, or the protein of the soybean, have been used to replace casein, forming similar "artificial ivories."

Deaminized Proteins.—Deaminized proteins, prepared by the action of nitrous acid on the protein, have been rather extensively studied. The resulting products contain no free $-NH_2$ groups and show quite altered chemical properties toward acids. So far as their hydrolytic products are concerned, the only notable change is that lysine can no longer be isolated, the reaction between the protein and the nitrous acid resulting in the elimination of the ϵ-amino group.

Deaminized proteins when fed to experimental animals produce a nutritional anemia. This is not a deficiency anemia, since supplementing the diet with good protein does not relieve the symptoms, but a toxic anemia.[106] Alcoholic sodium hydroxide extracts the toxic factor, leaving a relatively non-toxic residue. The nature of the toxin has not been elucidated.

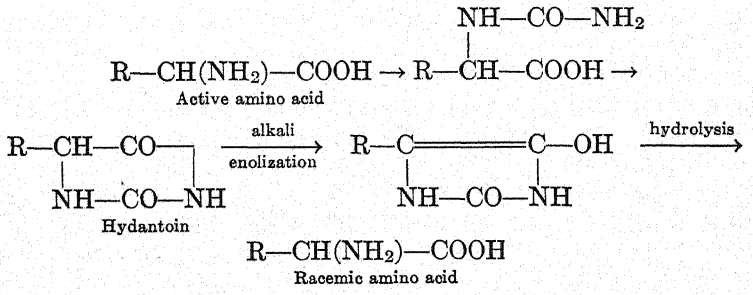
The Proteans.—The change of certain globulins to an insoluble modification, known as a protean, has already been noted. Edestin

[105] Brother, G. H., Casein Plastics, Chapter VI., in Sutermeister's Casein and Its Industrial Applications, Chemical Catalog Company, New York (1927).

[106] Smith, M. I., and Stohlman, E. F., The Anemia of Deaminized Casein, *U. S. Pub. Health Repts.*, 51: 772–786 (1936).

of hempseed, excelsin of the Brazil nut, and myosin of the muscle fibers are readily transformed from a protein which is easily peptized by 10 per cent sodium chloride to proteans which are no longer peptized. Here, again, we do not know the nature of the chemical change involved. Osborne [107] believes that this transformation involves hydrolysis and that the edestan may be regarded as the first stage in the hydrolysis of edestin. He finds that the transformation is accelerated by the presence of acids and that the various acids produce in a given time very different amounts of the edestan. The figures which he gives indicate that the hydrogen ion is the active catalyst, although his work was done prior to the general use of hydrogen-ion control. The chemical analysis of edestan is within experimental error of the chemical analysis of the edestin from which it was derived.

Racemized Proteins.—Dakin [108] observed that, when proteins were dissolved in dilute alkali, there was a progressive fall in the specific optical rotation with time, to a much lower value which eventually became constant. He observed a similar change in the optical rotation of hydantoins prepared from optically active amino acids and noted that, when hydantoins which had stood for a time in an alkaline solution were hydrolyzed, they yielded racemic rather than the active amino acids. He explains the change as a keto $\rightleftharpoons$ enol isomerism, the hydrogen migrating from the α-carbon atom to the carbonyl group, forming a double bond, as shown in the accompanying structural formulas. Later when hydrolysis occurs and the amino acids are regenerated, the double bond no longer exists, but since either one of the unions of the double bonds may later become attached to hydrogen, we would expect to find not the active but equal amounts of *d*- and *l*-acids to be formed.



In working with gelatin, Dakin found that a similar racemization occurred and that when the gelatin was later hydrolyzed, certain of

[107] Osborne, T. B., Ein hydrolytisches Derivat des Globulins Edestin und sein Verhältniss zu Weyl's Albuminat und zur Histongruppe, *Z. physiol. Chem.*, 33: 225–239 (1901).

[108] Dakin, H. D., The Racemization of Proteins and Their Derivatives Resulting from Tautomeric Change. Pt. I, *J. Biol. Chem.*, 13: 357–362 (1912).

the amino acids were optically inactive, whereas others were optically active. The inactive amino acids were leucine, aspartic acid, arginine, histidine, and phenylalanine. Part of the alanine was active; part was inactive. All of the proline, glutamic acid, and lysine which he isolated was optically active. He believes that the reason that certain amino acids were racemized, whereas others were not, depends upon the position of the amino acid in the protein molecule, and notes that only those amino acids, where both the $—NH_2$ group and the —COOH are combined in the protein molecule, undergo racemization. According to this viewpoint, the inactive amino acids are situated somewhere in the interior of the chain making up the protein molecule, whereas those which are not racemized are on the ends of the peptide chain.

Later, Dakin and Dale [109] compared the albumins of hens' eggs and ducks' eggs in regard to racemization and to acids which they yielded on hydrolysis. Only three amino acids were found to show characteristic differences. Leucine from racemized albumin from hens' eggs was almost completely racemic, whereas leucine isolated from the racemized duck albumin was almost completely active. Aspartic acid from the duck albumin was completely inactive, whereas that from hen albumin was partly active. Histidine from hen albumin was completely inactive, from duck albumin almost completely active. They believe that these observations indicate structural differences in the two albumins.

Woodman [110] investigated the corresponding proteins in cow and ox serum and in cow's milk and colostrum. He concludes that the globulins of cow and ox serum and of colostrum are identical, that the albumin of milk is identical with the albumin of colostrum, but that lactalbumin and serum albumin are not identical.

Dakin and Dudley [111] studied racemized casein, and Dudley and Woodman [112] repeated the work of Dakin and Dudley, comparing casein from cow's milk and casein from the milk of sheep. In casein from cow's milk, all the amino acids isolated were racemized, with the exception of proline and part of the valine and leucine. In casein from sheep's milk, lysine, proline, and tyrosine, were all optically active,

[109] Dakin, H. D., and Dale, H. H., Chemical Structure and Antigenic Specificity. A Comparison of the Crystalline Egg-Albumins of the Hen and the Duck, *Biochem. J.*, 13: 248–257 (1919).

[110] Woodman, H. E., A Comparative Investigation of the Corresponding Proteins of Cow and Ox Serum, Cow's Colostrum and Cow's Milk by the Method of Protein Racemisation, *Biochem. J.*, 15: 187–201 (1921).

[111] Dakin, H. D., and Dudley, H. W., The Racemization of Proteins and Their Derivatives Resulting from Tautomeric Change. Pt. II. The Racemization of Casein, *J. Biol. Chem.*, 15: 263–269 (1913).

[112] Dudley, H. W., and Woodman, H. E., The Specificity of Caseinogens. A Comparative Study of the Caseinogens of the Cow and the Sheep, *Biochem. J.*, 9: 97–102 (1915).

glutamic acid, leucine, and valine partly active. They, therefore, reached the conclusion that cow and sheep caseins contained the amino acids linked in different positions.

Dakin and Dudley [113] attempted to isolate tryptophane from racemized casein by tryptic digestion and found that neither trypsin, erepsin, nor pepsin would attack the racemized protein; neither was it digested *in vivo*, and when fed to dogs, it was eliminated unchanged in the feces. The racemic casein was not attacked by putrefactive bacteria during a 10-day interval, but after that was very slowly attacked. They point out that all artificial polypeptides which have been synthesized have been subjected at some stage or other to alkaline conditions, and suggest that possibly the failure of enzymes to attack certain of these synthetic products may be due to some change similar to the change which has taken place in protein racemization. They also note that, in the separation of proteins from biological materials, precautions must be taken not to subject them to the presence of alkalies for any extended time.

Ten Broeck [114] prepared racemic egg albumin by allowing egg albumin to stand at 37° for 3 weeks in contact with 0.5 *N* sodium hydroxide. He states that the product which was isolated differed chemically from egg albumin only in rotatory power. He found that this racemic protein had no immunological power. It neither sensitized nor intoxicated; no antibodies were formed. These observations support Vaughan's idea (*vide infra*) that a splitting of the protein must take place before immunological reactions occur. Obviously, if racemic proteins are not attacked by enzymes, no splitting can take place.

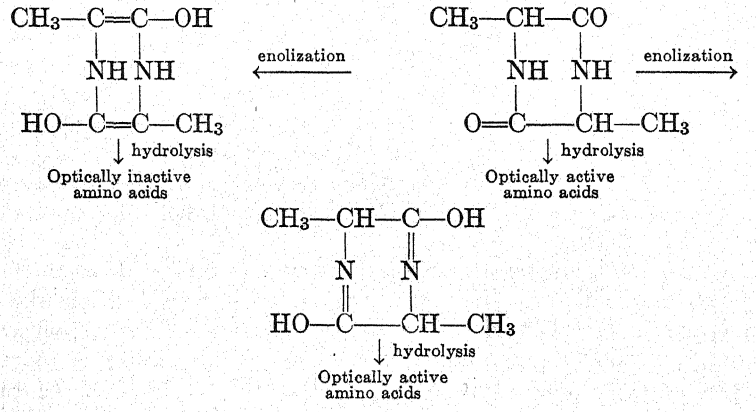
The above discussion and the papers cited would lead one to believe that a racemic protein is merely a protein in which certain shifts in linkages have taken place with a concomitant change in optical rotation, and that racemic proteins are somewhat analogous to coagulated proteins and proteans. However, this appears to be the wrong viewpoint. In most instances, the racemic product which is isolated amounts to only a fraction of the weight of protein which is originally taken. Thus, 20 to 30 grams of racemic casein can be isolated if 100 grams of casein have been acted upon by the 0.5 *N* sodium hydroxide. A large part of the casein has undergone deep-seated hydrolysis, and one of the hydrolytic products, *i.e.*, the racemized "protein," is still capable of being readily isolated. The question arises, therefore, whether the chemical analysis of this protein fraction can be compared with the chemical analysis of the original

[113] Dakin, H. D., and Dudley, H. W., The Action of Enzymes on Racemized Proteins, and Their Fate in the Animal Body, *J. Biol. Chem.*, 15: 271–276 (1913).

[114] Ten Broeck, C., The Non-Antigenic Properties of Racemized Egg Albumin, *J. Biol. Chem.*, 17: 369–375 (1914).

protein, in drawing valid conclusions. Csonka and Horn [115] show that rapid hydrolysis of the protein takes place in 0.5 *N* NaOH at room temperature and that the digestion products are optically active in the NaOH solution. However, when they are later boiled with 20 per cent NaOH to effect a complete hydrolysis, optical activity disappears. They suggest the discontinuance of the name "racemic protein."

Levene and Pfaltz [116] investigated the action of alkali on diketopiperazines and polypeptides. They find that the dipeptide, *d*-alanyl-*d*-alanine, is not racemized by alkali. The anhydride, however, changes from an optical rotation of $+17.5°$ to $-16.0°$, at which point 50 per cent of the nitrogen is amino nitrogen, *i.e.*, hydrolysis of the polypeptide has taken place. They note that the diketopiperazine ring must remain in contact with the alkali for an appreciable period of time before it hydrolyzes, in order to form intermediate products which on hydrolysis yield optically inactive acids. It will be noted that there are two enol forms of the dimethyldiketopiperazines, as shown in the following diagram, only one of which will yield the optically inactive amino acids.



Levene and Pfaltz note that even when the tripeptide, glycyl-*l*-alanylglycine, and the tetrapeptide, glycylglycyl-*l*-alanylglycine, were allowed to stand in contact with alkali, no racemization occurred during the first 48 hours, but on long standing, there was a slight decrease in optical rotation, the maximum racemization never exceeding 10 per cent and even in these cases hydrolysis reached 80 per cent or more. They note that there is no way of determining whether race-

[115] Csonka, F. A., and Horn, M. J., Studies on the Effect of Alkali on Protein I. The Optical Behavior of "Racemic Protein," *J. Biol. Chem.*, 93: 677–648 (1931).

[116] Levene, P. A., and Pfaltz, M. H., Studies on Racemization. Action of Alkali on Dextro-Alanyl-Dextro-Alanine Anhydride, *J. Biol. Chem.*, 63: 661–668 (1925); Studies on Racemization, III. Action of Alkali on Glycyl-Levoalanyl-Glycine and on Glycyl-Glycyl-Levoalanyl-Glycine, *ibid.*, 68: 277–283 (1926).

mization precedes or follows the hydrolysis, and point out that perhaps the racemization of proteins may be the racemization of diketopiperazine compounds rather than the long peptide chains which Dakin postulates. In considering the diketopiperazine theory of protein structure, we have already noted the later studies of Levene and Bass on the racemization of polypeptides, diketopiperazines, and proteins where they found that casein did not behave like either the polypeptides or the diketopiperazines. The rate of racemization was more nearly what one would expect for diketopiperazines. The rate of hydrolysis was more nearly what one would expect for polypeptides. They conclude that in casein either the diketopiperazine rings are unusually stable or the order of the linkage favors racemization.

Acid and Alkali Albumins.—These are perhaps best typified by Paal's [117] protalbinic and lysalbinic acids which have already been noted as examples of protective colloids. We should perhaps add to this group the products prepared by Vaughan's method [118] of partially hydrolyzing proteins in alcoholic solution with sodium hydroxide. In this way, proteins can be separated into two fractions, an alcohol-soluble portion and an alcohol-insoluble residue. The alcohol-soluble portion was found by Vaughan to be exceedingly poisonous when injected intravenously, exceeding in its toxic action even such drugs as strychnine. No adequate explanation for the high degree of toxicity has been offered. Vaughan notes, however, that gelatin appears to be the only protein which does not yield the toxic fraction. We have already noted that gelatin is deficient in a number of amino acids, those containing aromatic nuclei, and that it does not induce the usual immunological reactions. Vaughan believes the immunological reactions are, at least in part, induced by a hydrolytic cleavage in the animal body, more or less similar to the hydrolytic cleavage which takes place in an alcoholic sodium hydroxide solution, and that the toxins liberated in the anaphylactic reaction are similar, if not identical, to his protein poisons. Similar protein poisons [119] have been prepared from the proteins of the tubercle bacillus.

Relatively little work from the chemical standpoint has been done with these products. Miss Wheeler [120] has shown that they are complex, and has isolated a number of amino acids from the various preparations.

[117] Paal, C., Über die Einwirkung, ätzender Alkalien auf Eialbumin, *Ber.*, 35: 2195–2206 (1902).

[118] Vaughan, V. C., and Novy, F. G., Cellular Toxins, Lee Brothers (1902).

[119] White, B., and Avery, O. T., The Action of Certain Products Obtained from the Tubercle Bacillus. A. Cleavage Products of Tuberculo-Protein Obtained by the Method of Vaughan. I. The Poisonous Substance, *J. Med. Research*, 26: 317–356 (1912).

[120] Wheeler, S. M., A Study of the Chemistry of Bacterial Cellular Proteins, *J. Biol. Chem.*, 6: 509–552 (1909).

CHAPTER XVIII

THE REACTIONS OF PROTEINS WITH ACIDS AND BASES

From the preceding discussions of the chemical nature of the proteins, we have seen that the protein molecule possesses both acidic and basic groups and accordingly must be looked upon as a complex colloidal amphoteric substance. We have likewise noted that changes in hydrogen-ion concentration in many instances bring about molecular rearrangements, such as a shift in the keto $\rightleftharpoons$ enol isomerism, whereby additional reactive groups are formed within the protein molecule. We have similarly noted that proteins in solution show the characteristic properties of lyophilic sols and that the colloidal micelles may be either positively or negatively charged, depending upon the hydrogen-ion concentration of the dispersions medium.

It is not surprising, therefore, to find that proteins undergo reactions with acids and bases, and with certain salts, notably the salts of the heavy metals. These reactions have been studied by a great many workers and have given rise to a very voluminous literature. In order to discuss this literature adequately, it would require far more space than can be justified in the present connection. Casual mention can be made of only a few of the more important considerations.

In spite of the large amount of work which has been carried out in this field, the various workers are far from agreement. The workers may be classified by and large into three groups.

One group of workers insists that the reactions that take place between proteins and acids, bases, or salts are purely chemical reactions determined by primary valence forces of the free amino groups, the free carboxyl groups, or similar active groups within the protein molecule, and that the reactions are stoichiometrical in character, with colloidal reactions playing no appreciable role. This school of thought received a great impetus from the work of Loeb and is being ably continued since his death, through the activities of E. J. Cohn, D. I. Hitchcock, John Arthur Wilson, and C. L. A. Schmidt, to mention only a few of the outstanding workers.

Another group of workers view the reactions of proteins as the reactions of a lyophilic colloid system and support the view that the hydrogen ions, the hydroxyl ions, and the ion interchange with salt solutions is probably largely due to the forces of adsorption characteristic of lyophilic colloid interfaces. To this group the colloidal proper-

ties of the system far outweigh the chemical forces of primary valence.

A third group of workers may well include those who, though admitting that the forces of primary valence play a major role, nevertheless insist that the colloidal properties of a lyophilic system must be taken into account before an adequate knowledge of protein chemistry can be secured. This group insists that at least under certain conditions the colloidal forces outweigh the chemical forces of primary valence and tend in any event to modify the forces of primary valence.

Cohn [1] and Schmidt [2] have presented able reviews of the physical chemistry of the proteins, emphasizing in particular the viewpoint that the reaction between proteins and acids and bases are stoichiometrical reactions determined by the forces of primary valence.

We have already noted in the consideration of the Donnan equilibrium that the establishment of such an equilibrium is independent of the nature of the process which causes the formation of a non-diffusible ion. Similarly, it is immaterial in many instances whether a charged particle is formed by the process of ionization or by the colloidal process of adsorption. In either instance the charged micelle will behave as an ion, and as such will obey the physicochemical laws characteristic of an ionized system. It is this fact that makes it difficult to decide between the colloidal viewpoint and the purely stoichiometrical viewpoint in regard to protein compounds.

The author has supported the view that protein systems must from the very nature of the system show the typical reactions that are characteristic of lyophilic colloid systems, admitting at the same time that, if a protein is characterized by possessing free $-NH_2$ and free —COOH groups, such groups may be expected to react chemically with acids and bases, respectively. The newer viewpoint that the amino and carboxyl groups in amino acids, and presumably also in proteins, are present in the zwitterion form and that amino acids and proteins as zwitterions should show salt-like properties rather than the properties of acids and bases, should modify to some extent the older conceptions of acid-base binding relationships. One should not expect very weak bases to displace the hydrogen from the ammonium group of the zwitterion, nor should one expect very weak acids to repress the ionization of the potential carboxyl group of the zwitterion. Glycine apparently does not form a compound with ammonia, and most amino acids do not form salts with the fatty acids, *e.g.*, acetates with acetic acid. This is what one would expect from their zwitterion constitution. There seems accordingly no *a priori* reason why proteins, if the amino

[1] Cohn, E. J., The Physical Chemistry of the Proteins, *Physiol. Rev.*, 5: 349–437 (1925); *Ergeb. Physiol.*, 33: 781–882 (1931).

[2] Schmidt, C. L. A., Some Aspects of the Physical Chemistry of Amino Acids and Proteins, Chapter VI in Harrow and Sherwin's Textbook of Biochemistry, W. B. Saunders Company, Philadelphia (1935).

and carboxyl groups are in the zwitterion form, should be expected to bind stoichiometrically appreciable quantities of either ammonia or the weaker organic acids. With the stronger acids, such as hydrochloric acid, or the stronger bases, such as the fixed alkalies, one would anticipate a destruction of the zwitterion structure and a stoichiometric binding of acid or base equivalent to the number of zwitterions which were affected. Gliadin does unite [3] with acetic acid to form a "compound" in which 1 gram of gliadin binds a constant amount of 15.4 mg. acetic acid. This acetic acid is so strongly bound that it cannot be removed by any amount of washing with water, or by exhaustive extraction with alcohol or ether. The gliadin-acetic acid complex in solution has a *p*H of 3.99 as contrasted with a *p*H of 6.50 for the gliadin sol after electrodialysis, indicating that *the acetic acid was not neutralized by basic groups present in the protein.* The acid can be completely removed by electrodialysis. This then would appear to be a clear-cut case of the formation of a "gliadin acetate," if it were not for the fact that *purified alpha-cellulose adsorbs approximately the same amounts of acetic acid and holds it equally tenaciously.*[4] In the alpha-cellulose there are no basic groups to react stoichiometrically with the acetic acid, so that there the mechanism must be an interfacial adsorption. It seems probable that the "gliadin acetate" is likewise an adsorption complex where the adsorption of the acetic acid is molecular rather than ionic. We have already seen [5] that the adsorption of the fatty acids on charcoal is likewise a molecular rather than an ionic adsorption, so that apparently gliadin behaves in acetic acid solution somewhat similarly to charcoal.

The earlier literature in regard to acid and alkali binding has been reviewed by Hoffman and Gortner [6] and extended to a comparative study of the prolamines, in which study casein and fibrin were used as reference proteins. The earlier workers on acid and alkali binding used various types of physicochemical technic. These may be divided as follows:

The first method is the direct method of *precipitating out a protein compound.* This has been used by a number of workers, particularly those who have been dealing with the calcium compounds of casein, casein being titrated with calcium hydroxide to some definite end point as determined by some particular indicator, and then alcohol added to the system until the casein, containing a certain amount of calcium,

[3] Sinclair, W. B., and Gortner, R. A., *Cereal Chem.*, 10: 171–188 (1933).

[4] Gortner, R. A., and McNair, J. J., Alpha-Celluloses from Different Wood Sources, *Ind. Eng. Chem.*, 25: 505–510 (1933).

[5] Frampton, V. L., and Gortner, R. A., *loc. cit.*; *cf.* also Linner, E. R., and Gortner, R. A., *loc. cit.*

[6] Hoffman, W. F., and Gortner, R. A., Physico-Chemical Studies of Proteins. I. The Prolamines—Their Chemical Composition in Relation to Acid and Alkali Binding, Colloid Symposium Monograph, Vol. 2, pp. 209–368 (1925).

precipitates. Using this method, one can obtain casein precipitates with different calcium contents, depending upon the indicator used. When litmus is the indicator, only approximately one-half as much calcium is precipitated with the casein as when phenolphthalein is the indicator. Accordingly it has been suggested that casein combines with different quantities of calcium to form the so-called "monocalcium caseinate," and the so-called "dicalcium caseinate."

The author believes that this method is incapable of yielding definite results. The indicator chosen will determine to a very considerable extent the amount of calcium hydroxide which must be added to a given amount of casein before the neutral point is reached. Accordingly it would be possible to precipitate an entire range of casein-calcium "compounds," depending upon the indicators which were chosen, the range being from pure casein containing no calcium to casein saturated with calcium and contaminated with precipitated calcium hydroxide. In addition, the "compound" precipitated with alcohol may or may not have a ratio of casein to calcium, identical with the "compound" present in the solution prior to the addition of the alcohol.

A second method of indicating the presence of protein compounds was by *dissolving water-insoluble substances in protein solutions.* Thus, casein decomposes calcium carbonate when ground with precipitated calcium carbonate in water solution. Edestin or casein dissolves a certain amount of water-insoluble alkaloids, such as strychnine, or certain proteins dissolve freshly precipitated copper hydroxide to form sols containing the protein and calcium, strychnine, or copper, respectively. Edestin will dissolve approximately 35 per cent of its weight of copper from freshly precipitated copper hydroxide. The question arises here whether these reactions are the reactions characteristic of the hydrogen ions formed by the dissociation of —COOH groups or whether they are, in part at least, the reactions characteristic of peptization. Still a third possibility is the formation of complex metallic compounds in which a chelate type of linkage binds the metal to the nitrogen groups of the peptide linkages or other amino acid residues.

It would seem to the author that we do not have a clear-cut effect of the hydrogen ion, particularly in view of such solubility effects as have been noted for salt solutions by Kruyt and Robinson (*loc. cit.*). The protein sols may act as peptizing agents and undoubtedly will act as protective colloids, thus favoring peptization, so that at least a part of the reactions between proteins and insoluble substances may well be attributed to the colloidal behavior of the protein system. This statement should not be taken to mean that the author does not recognize that there are free carboxyl groups in the proteins, or carboxyl groups in the zwitterion form, and that these play a role in such reactions. The only point that it is desired to emphasize is that no method as yet

available delimits such actions from reactions characteristic of colloid systems.

A third method which has been proposed is to study *precipitates formed by the interaction of two soluble substances,* one containing a protein radical. Thus, protein dispersed in hydrochloric acid, when added to a metallic phosphotungstate, precipitates a protein phosphotungstate complex. The latter is possibly a chemical compound. Equally possibly it is an adsorption complex. Again we have a method where we cannot sharply separate the colloidal and the stoichiometrical reactions.

Electrical conductivity studies were first used by Sjöqvist [7] when he added increasing amounts of protein to a constant amount of acid until a constant electrical conductivity was reached. Figure 135 shows certain of his results.

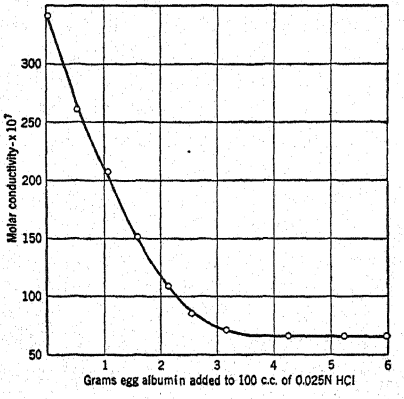


FIG. 135.—Showing the change in the electrical conductivity of a dilute hydrochloric acid solution to which increasing amounts of protein (egg albumin) were added. (Data of Sjöqvist.)

Hoffman and Gortner report similar studies for the prolamines and casein and fibrin. They were unable, however, to correlate their findings in any definite way with the chemical composition of the proteins which were studied.

More recently a number of conductivity studies have been carried out by Schmidt [8] and coworkers. These studies have shown that, in the case of sodium caseinate, the current is carried by the cations of the alkali metal and the casein anions and that ion transference experiments give about the same mobility for the casein ion as is indicated by conductivity measurements. When, however, the alkaline earths are combined with the casein, then abnormal values for the transference numbers are obtained, indicating that a considerable portion of the alkaline-earth metals were bound to the protein to yield negatively charged complex ions of the protein and the alkaline-earth residues. We know that in blood serum a considerable part of the

[7] Sjöqvist, J., Physiologisch-chemische Beobachtungen über Salzsäure, *Skand. Arch. Physiol.*, 5: 277–375 (1895); Berichtigungen und Zusätze zu meinem Aufsatz: Physiologisch-chemische Beobachtungen über die Salzsäure, *ibid.*, 6: 255–261 (1895).

[8] For literature see: Miyamoto, S., and Schmidt, C. L. A., Transference and Conductivity Studies on Solutions of Certain Proteins and Amino Acids with Special Reference to the Formation of Complex Ions Between the Alkaline Earth Elements and Certain Proteins, *J. Biol. Chem.*, 99: 335–358 (1933).

calcium is "bound" to the protein, so that it is held in a non-diffusible, non-ultrafilterable form and behaves as though it were actually a part of the protein micellar complex. Presumably other proteins behave similarly in forming these complex ions. Even in the case of solutions of the sodium salts of aspartic and glutamic acids, there is rather definite evidence [9] that ionic micelles are present. In the case of proteins there is no definite evidence that these ionic micelles are stoichiometrical chemical compounds any more than there is definite evidence that the ferric chloride in the ferric oxide hydrosols is bound to the ferric oxide in stoichiometrical relationships.

The cryoscopic method was first employed by Bugarszky and Liebermann,[10] who found that the addition of 6.4 grams of egg albumin to 100 cc. of 0.5 *N* hydrochloric acid reduced the depression of the freezing point of the acid solution to approximately 50 per cent of its initial value.

Barnett [11] made a comparative study of acid and alkali binding, using electrical conductivity, cryoscopic, and potentiometric technic, and reached the conclusion that the cryoscopic technic is the least valuable, owing to the small depressions contributed by the protein ions or micelles. In a number of instances the depression obtained was not appreciably greater than the experimental error of the method.

Another method is the *catalytic effect of hydrogen and hydroxyl ions.* Some reaction is selected which is catalyzed by one of these ions, for example, the inversion of sucrose by hydrogen ions [12] or the saponification of an ester by hydroxyl ions.[13] When a protein is added to such a system, the rate of catalysis is changed, and it is concluded that hydrogen ions have been removed from that system. Here, again, the method gives no indication of the mechanism whereby hydrogen or hydroxyl ions are removed from the solution.

Other methods have been proposed, such as *the masking of the toxicity of a heavy metal,* like barium, mercury, copper, or silver, by proteins, a higher concentration of these toxic metals being required when their solutions are mixed with proteins. Here, again, we

[9] Hoskins, W. M., Randall, M., and Schmidt, C. L. A., The Conductance and Activity Coefficients of Glutamic and Aspartic Acids and Their Monosodium Salts, *J. Biol. Chem.*, 88: 215–239 (1930).

[10] Bugarszky, S., and Liebermann, L. Über das Bindungsvermögen eiweissartiger Körper für Salzsäure, Natriumhydroxyd und Kochsalz, *Pflüger's Arch. ges. Physiol.*, 72: 51–74 (1898).

[11] Barnett, H. M., A Study of Acid and Base Binding by Proteins, thesis for the M.S. degree, filed in the Library of the University of Minnesota, Minneapolis (1927).

[12] Hoffman, F. A., Erkennung und Bestimmung der freien Salzsäure im Magensaft, *Z. klin. Med.*, 10: 793–796 (1889); *ibid.*, 11: 521–524 (1890).

[13] Wintgen, R., and Krüger, K., Über das Gleichgewicht Gelatine-Salzsäure, *Kolloid Z.*, 28: 81–89 (1921).

cannot distinguish between a chemical compound and an adsorption complex.

The method which has been most generally employed in recent years is the *potentiometric method,* involving the measurement of the change in hydrogen- or hydroxyl-ion concentration in acid or alkali solutions to which proteins have been added. This method was first used by Bugarszky and Liebermann,[14] who conclude that chlorine ions, as well as hydrogen ions, are bound by the protein. During the period 1900 to 1925 various workers [15] proposed equations by means of which the amount of acid or alkali bound by the protein could be calculated from hydrogen-ion-concentration measurements. All these equations, however, included the dissociation constant of the acid or alkali as calculated from conductivity measurements.

Hoffman and Gortner in their study of the prolamines measured the hydrogen-ion concentration of the acid and alkali solutions before and after the addition of protein and calculated the degree of ionization of the acid or alkali from the potentiometric data. The assumption was that a given hydrogen-ion concentration indicated a given normality of acid or alkali irrespective of the presence or absence of protein or protein micelles in the system. They pointed out that this assumption was probably not strictly correct but that no method of calculating the effect of the protein on the equilibrium was available. The equation which they used was

$$n = N - \frac{(H^+)}{\alpha'} \qquad (174)$$

where n = the amount of acid or alkali bound;
N = the original normality of the acid or alkali;
(H^+) = the hydrogen-ion concentration of the protein-acid or protein-alkali solution at equilibrium;
α' = the degree of ionization of the acid or alkali as determined by potentiometric methods.

Later, Cohn criticized this formula and proposed a new formula which in turn involves several assumptions. The derivation of his

[14] Bugarszky, S., and Liebermann, L., Über das Bindungsvermögen eiweissartiger Körper für Salzsäure, Natriumhydroxyd und Kochsalz, *Pflüger's Arch. ges. Physiol.,* 72: 51–74 (1898).

[15] Blasel, L., and Matula, J., Untersuchungen über physikalische Zustandsänderung der Kolloide. XVI. Versuche am Desaminoglutin, *Biochem. Z.,* 58: 417–450 (1913); Pauli, W., and Spitzer, A., quoted by Pauli, "Colloid Chemistry of the Proteins," Pt. I, pp. 93–95 (translated by P. C. L. Thorne), J. and A. Churchill, London (1922); Lloyd, D. J., and Mayes, C., The Titration Curve of Gelatine, *Proc. Roy. Soc. (London),* 93B: 69–85 (1922).

formula is given in a consideration of the base-binding capacity of casein.[16] Using the fundamental equation for deriving pH, *i.e.*,

$$\frac{\text{e.m.f. (observed)} - E\text{ (calomel electrode)}}{0.00019837T} = \log \frac{1}{H^+} = p\text{H} \qquad (175)$$

Cohn converts this into pOH by the equation,

$$p\text{H}^+ + p\text{OH}^- = p\text{K}_w \qquad (176)$$

which in turn can be converted into the logarithmic expression,

$$p\text{OH}^- = \log \frac{1}{(\text{OH}^-)} \qquad (177)$$

Cohn then introduces the activity coefficient γ in place of the dissociation coefficient α' of equation (174), the activity coefficient being determined by the ratio

$$\frac{(\text{OH}^-)}{(\text{NaOH})} = \gamma \qquad (178)$$

Combining equations (177) and (178), we have the equation,

$$p\text{OH}^- = \log \frac{1}{(\text{NaOH})} = p\text{NaOH} + p\gamma \qquad (179)$$

Table LXV shows the activity coefficients γ [17] and the logarithms of the reciprocals of the activity coefficients $p\gamma$ for varying concentrations of hydrochloric acid and sodium hydroxide.

In using equation (179) one must assume (*a*) that the sodium-protein compound is completely dissociated, (*b*) that the sodium ions from the sodium-protein compound have the same activity as sodium ions in a sodium hydroxide solution, and (*c*) that the protein ion does not influence this activity or rather that the influence of the protein ion on the activity of the sodium ion is the same as is the influence of an hydroxyl ion. Similarly, if equation (179) were used to study acid-binding, one would have to assume (*a*) the complete dissociation of the so-called "protein chloride" with the same activity coefficient for the chloride ions of the "protein chloride" as for the chloride ions in an equivalent concentration of hydrochloric acid, and (*b*) that there is no adsorption of the acid in the molecular state which can give rise to hydrogen ions in the solution. The data on gliadin-acetic acid already cited show that this assumption is probably unjustifiable. Various

[16] Cohn, E. J., and Berggren, R. E. L., Studies in the Physical Chemistry of the Proteins. III. The Relation between the Amino Acid Composition of Casein and Its Capacity to Combine with Base, *J. Gen. Physiol.*, 7: 45–79 (1924).

[17] Lewis, G. N., and Randall, M., Thermodynamics and the Free Energy of Chemical Substances, McGraw-Hill Book Company, New York (1923).

TABLE LXV

THE ACTIVITY COEFFICIENT γ (LEWIS AND RANDALL) AND THE LOGARITHMS OF THE RECIPROCAL OF THE ACTIVITY COEFFICIENT $p\gamma$ FOR VARIOUS CONCENTRATIONS OF HYDROCHLORIC ACID AND SODIUM HYDROXIDE

Molarity	HCl		NaOH	
	Activity Coefficient, γ	$\log \frac{1}{\gamma}$, $p\gamma$	Activity Coefficient, γ	$\log \frac{1}{\gamma}$, $p\gamma$
0.001	0.98	0.009	0.98	0.009
0.002	0.97	0.013	0.97	0.013
0.005	0.95	0.022	0.95	0.022
0.010	0.92	0.036	0.92	0.036
0.020	0.90	0.046	0.89	0.051
0.025	....	(0.048)	....	(0.055)
0.030	....	(0.050)	....	(0.059)
0.040	....	(0.053)	....	(0.065)
0.050	0.88	0.056	0.85	0.071
0.100	0.84	0.076	0.81	0.091

workers have indicated that either there is not complete dissociation of the "sodium proteinates" and "protein chlorides" or both anions and cations are adsorbed by the protein. If such a viewpoint is correct, this method of calculation introduces errors which may be as serious as the errors introduced by the equations which have been previously employed.

Using the above equations, Cohn concludes that casein has a maximum base-binding capacity of approximately 0.0014 mole of sodium hydroxide per gram of casein which had never been exposed to greater alkalinities than those which exist in nature, whereas casein which has been prepared by more drastic treatment has a maximum base-binding capacity of 0.0018 mole of sodium hydroxide per gram, 1 mole of sodium hydroxide, therefore, combining with 735 grams of unaltered casein or with 535 grams of casein somewhat altered.

Cohn's studies of "maximum" base-binding capacity involved concentrations of sodium hydroxide ranging between 0.03 N and 0.05 N. Hoffman and Gortner in their studies covered the range from 0.0005 N to 0.50 N. It seemed desirable, therefore, to repeat[18] the work, using the technic and method of calculation exactly as suggested by Cohn. In this study a succinic acid-sodium hydroxide system was compared

[18] Robinson, A. D., Gortner, R. A., and Palmer, L. S., *J. Phys. Chem.*, 36: 1857–1881 (1932).

with casein-sodium hydroxide and paracasein-sodium hydroxide systems. In the succinic acid-sodium hydroxide system the experimental and theoretical maximum base-binding capacities coincided. Neither the casein- nor paracasein-sodium hydroxide systems showed maximum base-binding capacities, but it was found that the amount of base bound by the casein was dependent upon the equilibrium hydrogen-ion concentration. Even alanine showed a varying base-binding capacity in the presence of different normalities of sodium hydroxide. Incidentally this study also included the effect of neutral salts on the protein-base-binding reaction. The "salt" effect was very much more marked for the protein-base systems than is characteristic of simple acid-base systems, indicating that the reactions of a protein-base system cannot be calculated by applying equations derived from the reactions of simple acid-base systems.

The conclusion was drawn from this study that equation (179) is inadequate for the calculation of base-binding, or the "sodium proteinate" is not completely dissociated at the higher concentrations of alkali, or sodium hydroxide is "adsorbed" on the ionic micelles according to a typical adsorption isotherm.

Certain observations of Hitchcock [19] are in agreement with the above studies. He studied acid binding of gelatin and edestin at various concentrations of hydrochloric acid, using both the hydrogen electrode and the chloride electrode. In the case of gelatin he concludes that in 0.1 N hydrochloric acid the protein combines with a maximum of 9.4×10^{-4} equivalent of H^+ and 1.7×10^{-4} equivalent of Cl^- per gram protein. In the case of edestin the corresponding values are 13.4×10^{-4} equivalent of H^+ and 3.9×10^{-4} equivalent of Cl^- per gram protein. *It will be noted that in both of these systems Cl^-, as well as H^+, is bound, thus indicating that either the "protein chloride" is not completely dissociated or that hydrochloric acid is adsorbed on the "protein chloride."*

Thomas and Mayer [20] used a Zeiss-Löwe interferometer and found that the refractive indices of mixed solutions of gelatin and of hydrochloric acid are not additive, whereas the refraction of mixed solutions of "gelatin chloride" and of hydrochloric acid are additive. Accordingly, when a gelatin sol is titrated with an acid, there is an abrupt change in the slope of the curve at some particular

[19] Hitchcock, D. I., The Combination of Gelatin with Hydrochloric Acid. II. New Determinations of the Isoelectric Point and Combining Capacity of a Purified Gelatin, *J. Gen. Physiol.*, 12: 495–509 (1929); The Combination of Edestin with Hydrochloric Acid, *ibid.*, 14: 99–103 (1930).

[20] Thomas, A. W., and Mayer, C. W., Estimation of the Acid Combining Capacity of a Protein by Means of the Interferometer, *Proc. Soc. Exptl. Biol. Med.*, 25: 667–669 (1928).

acid concentration, which they believe indicates the amount of acid which must be added to gelatin to form the "gelatin chloride." Their curve is shown in Fig. 136. Here again the technic is not capable of distinguishing between stoichiometrical compound formation and an adsorption reaction.

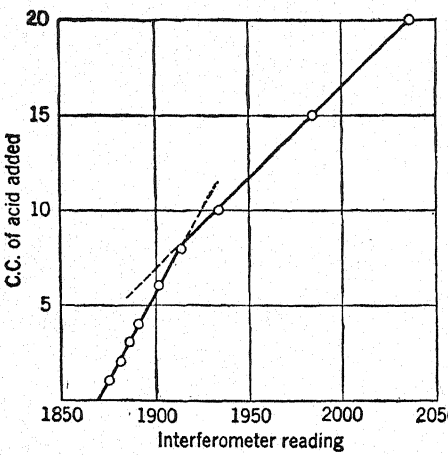


FIG. 136.—The titration curve of 0.881 gram gelatin with 0.1004 *N* HCl, volume 200 cc., as measured by readings in a liquid interferometer. (Data of Thomas and Mayer.)

The most recent technic and the one which appears to give the most valid information with respect to compound formation *vs.* adsorption reactions is that introduced by Bancroft and Barnett,[21] and later used by Belden,[22] and Czarnetzky and Schmidt.[23] In these studies, gaseous hydrochloric acid or gaseous ammonia is brought in contact with the solid protein, and the amount of gas which "combines" with the protein and the corresponding equilibrium pressure are measured. If the protein forms a solid "ammonium proteinate" or a solid "protein chloride," the equilibrium pressure of the gas must remain constant until all the protein has been converted into the protein compound. If ammonia or hydrogen chloride is adsorbed by the protein, there will be a smooth combination curve characteristic of adsorption.

When the phase rule

$$F = C - p + 2 \qquad (180)$$

is applied to this system of protein plus vapor, there will be three phases—protein, ammonium proteinate (or protein chloride), and ammonia (or hydrogen chloride)—with one degree of freedom. When the experiments are carried out at constant temperature, the degree of freedom is removed, and the system becomes invariant. In such a

[21] Bancroft, W. D., and Barnett, C. E., Phase Rule Studies on the Proteins. I. Determination of Solid Compounds with Hydrogen Chloride or Ammonia, *J. Phys. Chem.*, 34: 449–498 (1930); II–VII, *J. Phys. Chem.*, 34: 753–798, 1217–1253, 1930–1946, 2433–2471 (1930); *ibid.*, 36: 1285–1323, 2299–2305 (1932).

[22] Belden, B. C., Gelatin with Ammonia and Hydrogen Chloride, *J. Phys. Chem.*, 35: 2164–2193 (1931).

[23] Czarnetzky, E. J., and Schmidt, C. L. A., Studies on the Combinations of Certain Amino Acids and Proteins in the Solid State with Certain Gaseous Acids and Bases, *J. Biol. Chem.*, 105: 301–317 (1934).

case the pressure must remain constant, irrespective of the amount of gaseous component in the two solid phases, *i.e.*, until all the protein has been converted into the protein compound. When this point is reached, only two phases and two degrees of freedom are present, so that the pressure will change at constant temperature. If the protein adsorbs the gas, then the system is bivariant, since it consists of only two phases, and at constant temperature the pressure will vary continuously with the concentration of the gas in the solid phase. These conditions are shown diagrammatically in Fig. 137. In *A*, we have

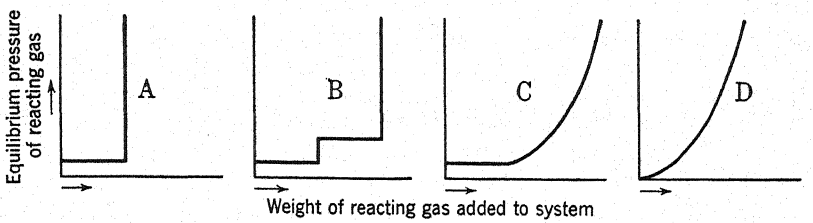


FIG. 137.—Showing diagrammatically the phase rule behavior of a gas reacting on a solid where (*A*) the solid is monoreactive and where the reaction is purely stoichiometric, (*B*) where the solid is direactive and the reaction is purely stoichiometric, (*C*) where the solid is monoreactive and the product formed by the stoichiometric reaction adsorbs the gas, and (*D*) where the interaction between the solid and the gas is wholly one of adsorption.

the gaseous titration of a solid capable of combining with one molecule of an acid (or base) to form a definite monobasic compound. In *B*, the solid combines with two molecules of an acid (or base) to form a dibasic compound. In *C*, a monobasic stoichiometrical reaction precedes additional adsorption of the acid (or the base) on the original compound which was formed; and in *D*, the solid adsorbs the acid (or the base) without definite compound formation.

As the result of his phase-rule studies Bancroft concludes that casein, zein, arachin, fibrin, and gliadin readily adsorb ammonia, but that there is no evidence for the formation of any chemical compound. Casein, arachin, fibrin, gliadin, and edestin formed definite compounds with hydrogen chloride, but the amount of hydrogen chloride which combined with these proteins was different from the amount found by previous investigators working with protein solutions and using potentiometric technics. In the dry state much larger quantities of acid combined. Bancroft found zein not to form a chemical compound with hydrogen chloride. All the protein chlorides adsorbed additional hydrogen chloride after compound formation had been completed (Fig. 137*C*). Zein adsorbed hydrogen chloride without compound formation (Fig. 137*D*).

Czarnetzky and Schmidt do not agree with Bancroft's findings with respect to compound formation between zein and hydrochloric acid.

Neither do they agree to the absence of compound formation in the case of casein, gelatin, edestin, and zein in the presence of ammonia. They report definite ammonium compounds. They also report a definite zein-hydrochloric acid compound but note that the amount of hydrochloric acid which is bound is exceedingly small. It is difficult to account for the divergence in the two series of data. The acid-binding capacity of casein and gelatin is of the same order of magnitude in Bancroft's studies and in those of Czarnetzky and Schmidt. In the case of edestin the acid-binding capacity in Bancroft's experiments is approximately twice that found by Czarnetzky and Schmidt, and the marked discrepancy in the two series of data in the case of zein has already been noted. Both groups of workers report that glycine forms no compound with ammonia. This would be anticipated if glycine is already a substituted ammonium salt existing in the zwitterion form. If the proteins used were in the zwitterion form, one would anticipate that they would not form compounds with ammonia. If in some way the zwitterion form had been destroyed, the compound formation with ammonia would be expected. It may be that the discrepancy in the two series of data is a reflection of the methods by which the proteins were originally prepared. In any event, Czarnetzky and Schmidt note that, "beyond the region of the ammonia or hydrogen chloride-protein titration curves where chemical combination has taken place, the curves are generally not, as might be expected, straight lines. This is probably due to the fact that the relation between the solid phase and the gas is somewhat analogous to that which exists between other solids having large surfaces and certain gases." In other words, although they do not use the colloid chemist's terminology, *adsorption of ammonia or hydrogen chloride takes place on the surface of the ammonium proteinate or the protein chloride which is initially formed, and this is what Bancroft observed in his studies.* These experiments to which the phase rule can be rigidly applied, therefore, bear out the author's original contention that *chemical combination of proteins with acids or bases to form stoichiometrical compounds is complicated by colloidal adsorption reactions.*

CHAPTER XIX

THE DIGESTION AND METABOLISM OF PROTEINS

Man (and, so far as we know, every vertebrate) is absolutely dependent upon the plant kingdom for certain of the amino acids which he needs to synthesize into the proteins characteristic of his own tissues. Whether or not forms of animal life below the vertebrates are similarly dependent is still an open question, and there has been little investigational work in this field. *The vast majority of the amino-acid molecules in our bodies are derived from plant proteins where they are produced from their inorganic constituents by the action of photosynthesis.* To be sure, some of the amino acids in the proteins of our bodies may have been derived from the proteins of animals used by us as foods, but the proteins of these animals, in the last analysis, were composed of amino acids obtained from plants, and apparently they have been passed down to us unchanged in their chemical configuration.

In a series of brilliant researches Rose[1] has shown by feeding known mixtures of amino acids that *at least ten amino acids derived from food sources are necessary for the growth of the white rat. These ten amino acids are lysine, valine, tryptophane, histidine, phenylalanine, leucine, isoleucine, threonine, arginine, and methionine.* Rose lists those amino acids which were not found to be essential under his feeding conditions as glycine, alanine, serine, norleucine, aspartic acid, glutamic acid, hydroxyglutamic acid, tyrosine, cystine, citrulline, proline, and hydroxyproline. The feeding experiments were of relatively short duration (generally 24 days), so that they still leave open the problem as to whether additional amino acids are necessary for long-continued maintenance, for reproduction, and for lactation, and also whether a diet containing only the "essential" amino acids would shorten the usual life span. In most of the feeding experiments all or nearly all of the "non-essential" amino acids were included so as to limit the unknown factor to the one amino acid under investigation.

Whether or not man has an amino-acid requirement similar to that of the white rat we do not know, but it appears improbable that man can synthesize more amino acids than the white rat can, and therefore we can state that in all probability the ten essential amino acids noted

[1] Rose, W. C., *et al.*, Feeding Experiments with Mixtures of Highly Purified Amino Acids, I-VIII, *J. Biol. Chem.*, 94: 155–165, 167–171, 173–184 (1931); *ibid.*, 107: 45–55, 57–73, 449–458 (1934); *ibid.*, 112: 275–282, 283–302 (1935).

above are likewise essential for man, with the added possibility that some of those amino acids which are dispensable for the white rat may be essential for man. To conduct an experiment on man similar to those conducted by Rose on rats would involve at the present time a prohibitive cost. The difficulty, therefore, in proving which amino acids are essential and which are non-essential for the human organism lies in the difficulty in securing foodstuffs in which we can prove the presence or the absence of the particular amino acids. In many instances quantitative analytical methods for certain specific amino acids are either wholly lacking or are too crude to ascertain whether or not that amino acid is present in small amount or is completely absent in the protein which is fed.

Rose is following up the feeding studies with other investigations designed to ascertain the amount of each amino acid which must be present in the diet. At the 1937 meeting of the Federation of Biological Societies he reported [2] that each 100 parts of food should contain 0.6 per cent threonine, 0.5 per cent isoleucine, 0.7 per cent phenylalanine, 0.6 per cent methionine, 0.4 per cent histidine, 1.0 per cent lysine, 0.2 per cent tryptophane, 0.7 per cent valine, and 0.2 per cent arginine.

The utter dependency of the vertebrates upon the plant kingdom perhaps cannot be better expressed than by the statement that the vertebrate cannot even insert an $—NH_2$ group into the ϵ-carbon atom of α-amino caproic acid to form α-ϵ-diaminocaproic acid or lysine, this in spite of the fact that the animals were starving to death because there was no lysine in the diet, and were deliberately given [3] an adequate amount of α-amino caproic acid. Similar experiments have been conducted with other synthetic materials with essentially similar results, except that *d-l*-β-4-imidazolelactic acid, when fed [4] with diets deficient in histidine, caused a resumption of growth, indicating that an amino group had apparently been inserted in place of the —OH group of the lactic acid radical. This observation has been confirmed [5] with the further observation that imidazole pyruvic acid is able to some extent to replace histidine in the diet. Whether or not histidine actually is synthesized from imidazole pyruvic acid is still uncertain. It may be that no amino group is inserted in the substituted imidazole, that the β-imidazolelactic acid, *as such,* functions in place of

[2] Rose, W. C., The Nutritive Significance of the Amino Acids and Certain Related Compounds, *Science,* 86: 298–300 (1937).

[3] Lewis, H. B., and Root, L. E., Amino-Acid Synthesis in Animal Organism. Can Nor-Leucine Replace Lysine for the Nutritive Requirements of the White Rat, *J. Biol. Chem.,* 43: 79–87 (1920).

[4] Cox, G. J., and Rose, W. C., The Availability of Synthetic Imidazoles in Supplementing Diets Deficient in Histidine, *J. Biol. Chem.,* 68: 781–799 (1926).

[5] Harrow, B., and Sherwin, C. P., Synthesis of Amino Acids in the Animal Body. IV. Synthesis of Histidine, *J. Biol. Chem.,* 70: 683–695 (1926).

the histidine. Methylimidazole, hydroxymethylimidazole, imidazolecarboxylic acid, β-imidazolepropionic acid, and β-imidazoleacrylic acid did not supplement histidine-deficient diets.

Tyrosine apparently is formed in the animal organism from phenylalanine, but the animal organism apparently cannot form phenylalanine from tyrosine. Cystine, methionine, and homocystine (the next higher homolog, prepared synthetically by removing the methyl group from the sulfur of methionine, after which two molecules of the homocysteine combine to form homocystine), can apparently be used more or less interchangeably as one of the essential amino acids, although a minimal amount of methionine must be present and cannot be replaced by cystine. The organic chemistry involved in the problems of the interchangeability of these three amino acids is still uncertain.

While arginine can be synthesized by the animal organism, it cannot be synthesized at the rate required for *normal* growth, accordingly Rose lists it among the essential amino acids.

The modern ideas of protein nutrition are vastly different from those annunciated by Liebig. The old idea involved the presence of an ample supply of protein, carbohydrate, fat, and mineral matter. Later, physiologists introduced the qualification that a minimal amount of energy was required, and accordingly the calorific equivalent of the diet was added. The newer viewpoint, largely brought about by the pioneer researches of Osborne and Mendel, and McCollum and his co-workers, has shown that *protein quantity* is not an adequate criterion of diet, and that *protein quality* must be taken into consideration, *i.e.*, the question must be answered, "Does the protein which is eaten contain the essential amino acids in adequate amounts for normal growth and maintenance?" To use Dr. McCollum's illustration, if the body needs "Peter-Piper-picked-a-peck-of-pickled-peppers" protein, it will not secure normal nutrition but will starve to death on a "Theosophilus-Thistle-the-successful-thistle-sifter" protein.

We cannot go into the role played by the individual amino acids, nor into the experiments which prove them to be essential. Any modern textbook on nutrition will be found to cover these points. All that can be emphasized is the striking specificity of the biological processes involved both in growth and maintenance, as illustrated by the fact that if one essential amino acid is lacking from the diet, the entire growth mechanism may break down completely and the animal starve to death.

The whole viewpoint of the mechanism of protein metabolism within the animal body has changed within the last three decades almost as radically as have the ideas of what constitutes normal nutrition. The older workers supported the view that the proteins were digested by pepsin and hydrochloric acid in the stomach to proteoses and peptones and that these were further digested to peptides and

amino acids by the tryptic and ereptic enzymes of the duodenum and small intestine. It was then believed that the peptides were absorbed, as such, through the intestinal wall and were resynthesized into protein in the intestinal wall, passing into and being transported by the blood stream as proteins.

Van Slyke and Meyer [6] report the presence of *amino nitrogen* in the non-protein nitrogen fraction of the blood, and that the non-protein amino nitrogen in the blood increased with a meat diet, thus indicating that free amino acids were transported, as such, in the blood stream. However, the presence of amino nitrogen did not definitely prove the presence of the simple amino acids, for it may have been derived from relatively simple peptides or from other compounds.

We have already noted the vividiffusion apparatus of Abel, and so far as the author is aware, Abel should be credited with the first absolute proof that amino acids are transported, as such, in the blood stream. Shortly after the preliminary publication of Abel,[7] Abderhalden [8] reported the isolation of a considerable quantity of amino acids from blood, thus confirming Abel's observations by an independent method. These series of observations changed the earlier theories to the modern belief that the amino acids are transported as such in the blood stream to the various cells and tissues, where such as are needed are resynthesized into the proteins characteristic of those cells and tissues.

Gastric digestion by pepsin reduces proteins only to the stage of proteoses and peptones. Probably no simple amino acids are formed by gastric digestion, and probably no appreciable absorption takes place from the stomach. We can, therefore, look upon gastric digestion of protein as a preliminary hydrolysis and hydration, rendering the split products more susceptible to enzyme action after the food has passed into the duodenum.

In intestinal digestion, the proteins are attacked by trypsin in the upper portion of the intestines and by erepsin somewhat lower down. Both trypsin and erepsin hydrolyze proteins to their constituent amino acids. These amino acids then diffuse through the intestinal wall into the blood stream and are transported to the various cells and tissues of the body. At any one time there is an extremely small concentration of amino acids in the blood stream. It has been calculated that at the height of digestion the rate of blood flow through the portal

[6] Van Slyke, D. D., and Meyer, G. M., The Amino-Acid Nitrogen of the Blood. Preliminary Experiments on Protein Assimilation, *J. Biol. Chem.*, 12: 399–410 (1912).

[7] Abel, J. J., Rowntree, L. G., and Turner, B. B., On the Removal of Diffusible Substances from the Circulating Blood by Means of Dialysis, *Trans. Assoc. Am. Physicians*, 28: 51–54 (1913).

[8] Abderhalden, E., Der Nachweis von freien Aminosäuren im Blute unter normalen Verhältnissen, *Z. physiol. Chem.*, 88: 478–483 (1913).

vein in a 9.5-kg. dog is approximately 9,000 cc. per hour, or at a rate of 150 cc. per minute. Protein is only slowly digested and absorbed. Pflüger has stated that the absorption of 1.14 grams of protein per kilo of body weight per hour is a good absorption for a human being. Accordingly if such an absorption of protein were to take place in the dog noted above, the concentration of amino acids in the blood would not exceed 0.12 per cent. Some workers place this value as low as 0.005 per cent. It is not surprising, therefore, that, by the earlier and somewhat crude methods, the presence of amino acids in the blood stream was overlooked and that only the more refined technic of the vividiffusion or the Van Slyke apparatus was able to detect the presence of appreciable quantities of amino acids. Abderhalden in his isolation of amino acids from the blood stream worked up approximately 100 liters of blood and identified proline, leucine, valine, aspartic and glutamic acids, glycine, arginine, histidine, and lysine. No amino acid was found in amounts exceeding 0.40 gram.

It should be noted at this point that the older workers were justified in concluding that amino acids were not present, partly because of the inadequacy of their methods and partly because of the fact that certain other nitrogenous compounds, for example, urea, creatine, creatinine, uric acid, ammonia, etc., are always present in the blood serum from which the proteins have been removed by appropriate technic. Some of these constituents may well occur in much larger quantities than the amino acids, and accordingly unless very delicate technic were employed, it would be impossible to identify such traces of amino acids as are normally present during the absorption of protein.

It is perhaps pertinent to ask whether or not polypeptides, proteoses, peptones, and perhaps even proteins may not at times pass from the digestive tract into the circulation. The author believes that this is not only a possibility but a probability. Certain individuals and particularly infants develop pathological conditions which can be traced to the inclusion of some particular protein in the diet, in that they become exceedingly hypersensitive to some of these proteins. Egg albumin appears to be one of the proteins most commonly producing such hypersensitivity, although many cases have been noted where the proteins of the legumes, particularly peas, are involved. We have already noted that the egg-albumin molecules are so small that they will dialyze slowly through a fairly porous collodion membrane. It would seem probable, therefore, that conditions may arise whereby the membranes separating the intestinal contents from the blood stream become sufficiently permeable to permit the passage of small amounts of foreign proteins, thus giving rise to protein intoxication. Aside from its pathological significance this does not appear to be an important problem in protein assimilation.

No adequate theory has been advanced to account for the synthesis

of the vital proteins in the various cells and tissues from the heterogeneous mixture of amino acids which are transported in the blood stream. We only know that the vital process involved is one of rigid selection and that a given cell or tissue invariably synthesizes proteins containing certain specific amino acids linked in a certain specific way. We have no clue as to the mechanism whereby this selective action is brought about.

Alcock [9] has recently summarized our knowledge of the synthesis of proteins *in vivo*. He suggests that the idea is untenable that proteins are synthesized by an enzymatic condensation of the amino acids. Such a mechanism could not provide for specificity. He suggests that the synthesis of a primitive type of protein from relatively simple materials is a common attribute of all living organisms. We have already noted his urprotein, in the composition of which arginine, histidine, lysine, cystine, and tryptophane play prominent roles. His suggested process of synthesis is as follows: "The blood amino acids are taken up by the tissues, and broken down to some simple unit. This unit, by polymerisation, provides a basis on which the protein is built. Within this basic molecule a process of differentiation sets in and a definite proportion of each amino acid is formed, the protein so produced being similar in composition to the probably ancestral protein. The subsequent differentiation of the molecule, involving the addition or elimination of amino acids, or the addition of prosthetic groups, follows a course determined by the nature of the tissue in which it is being produced."

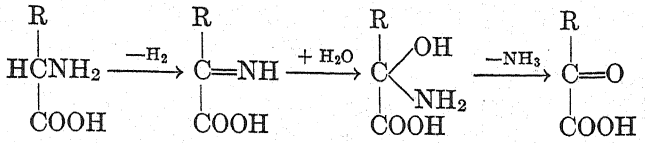
It is obvious from this quotation that we know practically nothing about the synthesis of proteins by living organisms.

THE DEGRADATION OF AMINO ACIDS IN THE BODY.—The body apparently possesses a very efficient mechanism for maintaining a low concentration of amino acids in the blood stream even when protein digestion and absorption are at a maximum. This mechanism resides chiefly in the liver where the amino acids are deaminized and broken down, the nitrogenous portion being eliminated as urea by the kidneys.

The principal mechanism of deamination of amino acids in the animal body is apparently an oxidative deamination giving rise to an α-ketonic acid and ammonia as end products. This mechanism was originally proposed by Knoop.[10] It apparently proceeds in three stages:

[9] Alcock, R. S., The Synthesis of Proteins *in Vivo*, *Physiol. Rev.*, 16: 1–18 (1936).

[10] Knoop, F., Über den physiologischen Abbau der Säuren und die Synthese einer Aminosäure im Tierkörper, *Z. physiol. Chem.*, 67: 489–502 (1910); *cf.* also Embden, G., and Schmitz, E., Über synthetische Bildung von Aminosäuren in der Leber, I and II, *Biochem. Z.*, 29: 423–428 (1910), 38: 393–406 (1912); Kondo, K., III, *ibid.*, 38: 407–413 (1912).



the first of which is catalyzed by dehydrogenases and which requires an accompanying hydrogen acceptor, since hydrogen acceptors and dehydrogenases are usually a part of an oxidation $\rightleftharpoons$ reduction cycle in which atmospheric oxygen is involved. Nearly all dehydrogenation reactions are accompanied by utilization of oxygen. The deamination process is no exception to this rule, for it fails under anaerobic conditions.

α-Ketonic acids corresponding to many of the amino acids have been isolated as would be required by Knoop's theory. More interesting still, Knoop observed that the sequence of reactions noted above was reversible and that *α-amino acids could be synthesized in the animal organism by perfusing the liver (dog) with the corresponding α-keto acid.* Thus, tyrosine and tyrosine derivatives were isolated by perfusing with defibrinated blood to which the ammonium salt of *p*-oxyphenylpyroracemic acid had been added. Similarly alanine, phenylalanine, leucine, α-amino-*n*-butyric acid, and norleucine were prepared by perfusing with the ammonium salts of the corresponding α-keto acid. In the case of the last two compounds, the author notes that they are not naturally occurring amino acids. Since that observation both of these compounds have been reported as occurring in proteins. Still more interesting, *the amino acids which were synthesized by perfusing with the ammonium salt of the α-keto acid were optically active.*

In addition to this principal reaction there are several other deamination possibilities which may occur to a minor extent. Thus, *simple deamination* might yield a ketonic aldehyde,

$$R—CH(NH_2)COOH \rightarrow R—CO—CHO + NH_3$$

or the reaction may involve the elements of water and result in a *hydrolytic deamination*, with the formation, as the primary product, of an α-hydroxy acid,

$$R—CH(NH_2)COOH + H_2O \rightarrow R—CH(OH)—COOH + NH_3$$

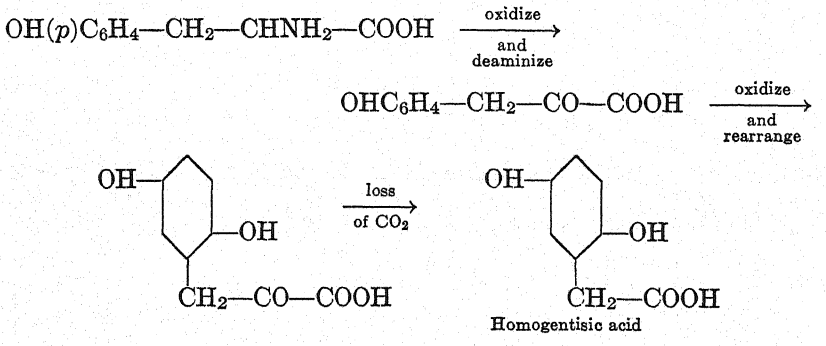
or there may be an *oxidative deamination* resulting in a saturated acid containing one less carbon than the original amino acid,

$$R—CH_2—CH(NH_2)COOH + O_2 = R—CH_2—COOH + NH_3 + CO_2$$

Occasionally, under pathological conditions, the oxidation of amino acids does not follow the normal process. In such cases we have the formation not of α-ketonic acids but of β-ketonic acids. These are very resistant to further oxidation. Such types of oxidation are characteristic of diabetes where appreciable quantities of β-oxybutyric acid and of acetoacetic acids may be formed. The presence of any appreciable quantities of these β-ketonic acids (derived from either protein or fat) is indicative of a serious pathological condition.

The amino acids containing an aromatic nucleus apparently undergo the α-ketonic oxidation, and there is some evidence that a part of the aromatic nuclei may be opened and burned within the animal body. However, the phenols which are normal constituents of the urine, and indole, skatole, indigo blue, etc., appear to have their origin in the benzene and indole rings of the amino acids by action of the intestinal bacteria.

Under certain conditions the mode of oxidation of certain of the amino acids appears to be altered within the animal body, tyrosine, for example, giving rise to homogentisic acid, probably according to the following scheme:



The homogentisic acid appears in the urine in certain diseases, such as melanouria and alkaptonuria, the urine being colorless when voided and rapidly darkening to an intense black liquid from which black particles are precipitated.

The presence of homogentisic acid in the urine should not be taken necessarily as an indication of a pathological condition. This mechanism for the oxidation of tyrosine and phenylalanine appears to be hereditary, is more prevalent in males than in females, and may persist throughout life without any evidence of harmful effects. An increased consumption of proteins containing tyrosine or phenylalanine will increase the amount of homogentisic acid which is excreted. Rather surprisingly *p*-hydroxyphenollactic acid when ingested does not

affect the amount of homogentisic acid which is excreted. The corresponding keto acid is excreted as homogentisic acid, indicating that hydrolytic deamination probably does not take place normally to any great extent.

The sulfur of cystine on oxidation in the body yields sulfuric acid and sulfates. Again, in this instance, cystineuria may occur, the cystine being eliminated as such in the urine. This again need not necessarily be taken as indication of a pathological condition, inasmuch as it is hereditary, affecting males and females about equally. The only untoward symptom which may arise is the aggregation of the cystine crystals into kidney stones or urinary concretions. Cystine is so insoluble that there are a number of instances on record where such concretions have been formed. Thus, Tennant [11] reports a surgical case in which 15 stones, having a total weight of 73 grams, were removed from a kidney. These stones contained [12] 93 per cent of pure cystine.

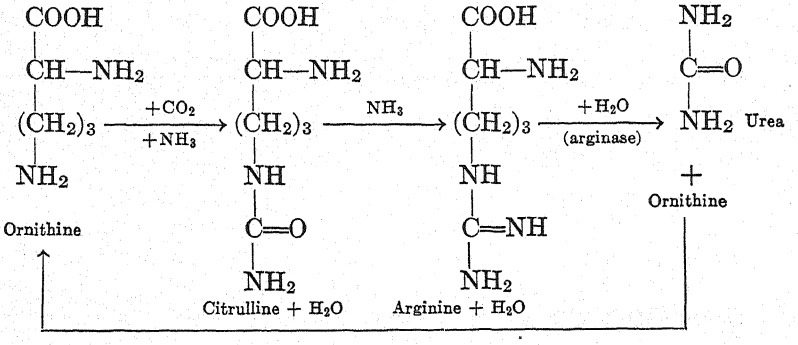
UREA FORMATION.—Until recently it had been assumed that urea arose in the animal body, first through the condensation of ammonia and carbon dioxide to form ammonium carbonate, and this with the loss of water formed the intermediate ammonium carbamate, and this further condensed to form carbamide, or urea. An alternative hypothesis was the formation of ammonium cyanate which rearranged to form urea. The difficulty with either one of these theories was that, in the mammal, urea synthesis appears to reside wholly in the liver and all efforts have failed to link urease, the enzyme which decomposes urea into ammonia and carbon dioxide, with the synthetic mechanism.

In 1932, Krebs and Henseleit [13] demonstrated a new mechanism for the synthesis of urea in the mammalian liver which appears to be the long-sought synthetic mechanism. This involves a cycle in which ornithine combines with one molecule of ammonia and one molecule of carbon dioxide with the loss of a molecule of water to form citrulline. The citrulline then combines with an additional molecule of ammonia with the loss of a molecule of water to form arginine. Arginine in the presence of water is hydrolyzed by the enzyme arginase to form one molecule of ornithine and one molecule of urea. The ornithine which is thus regenerated is available for again starting a new cycle.

[11] Tennant, C. E., Cystin Calculi: A Complex Surgical Problem, *J. Am. Med. Assoc.*, 80: 305–307 (1923).

[12] Gortner, R. A., and Hoffman, W. F., A Chemical Study of Cystine from Kidney Stones, *Proc. Soc. Exptl. Biol. Med.*, 23: 691–693 (1926).

[13] Krebs, H. A., and Henseleit, K., Untersuchungen über die Harnstoffbildung im Tierkörper, *Z. physiol. Chem.*, 210: 33–66 (1932).



Krebs and Henseleit incubated ammonium carbonate with macerated tissue from seventeen mammalian organs, and in their experiments liver was the only organ which converted the ammonium carbonate into urea. They found that both ornithine and citrulline had remarkable catalytic activity in favoring urea synthesis. Later Manderscheid [14] found that the same mechanism holds for amphibia and reptiles so far as these animals form urea. In birds where the end product is uric acid, the failure of the mechanism appears to be due to the absence or limiting amounts of arginase in the liver.

The end products of protein metabolism, allantoin and urea, have taken on an added interest since the observations of Robinson.[15] He was investigating the reasons for the beneficial effects in the healing of chronic ulcers which have been cleaned by maggot therapy. During the World War it was observed that certain suppurative wounds which had become infected with blow-fly maggots healed much more rapidly than similar wounds in which the maggots were absent. This has given rise to the use of maggots in cleaning suppurative wounds, the belief being that the only function of the maggots was to clean the wound by devouring dead tissue. However, it was observed that if the maggots were removed from the wound after they had completely cleaned it, the healing process slowed down. On the other hand, if the maggots were left in contact with the cleaned wound, the healing was remarkably accelerated. Inasmuch as no secretions of the blow-fly maggot are known, Robinson investigated the excretory products. One of these he found to be allantoin, and when wet dressings satu-

[14] Manderscheid, Hildegard, Über die Harnstoffbildung bei den Wirbeltieren, *Biochem. Z.*, 263: 245–249 (1933).

[15] Robinson, William, Stimulation of Healing in Non-healing Wounds by Allantoin Occurring in Maggot Secretions and of Wide Biological Distribution, *J. Bone and Joint Surgery*, 17: 267–271 (1935); Use of Urea to Stimulate Healing in Chronic Purulent Wounds, *Am. J. Surgery*, 33: 192–197 (1936); *cf.* also Holder, H. G., and MacKay, E. M., The Use of Urea in the Treatment of Infected Wounds, *J. Am. Med. Assoc.*, 108: 1167–1169 (1937).

rated with a solution of allantoin were placed in contact with the clean wound, healing was remarkably accelerated. Later, after crystallizing out the allantoin from the excretory products of the maggots, Robinson found the residual solution still to have healing activity. The residual nitrogenous constituents were largely urea, and on testing urea as a healing agent, it likewise was found to stimulate the healing process and to promote the development of granulation tissue. Many suppurative wounds and chronic ulcers of long standing have been healed by means of either allantoin or urea.

Macalister [16] had earlier noted the healing properties of allantoin and had suggested its use in the treatment of chronic ulcers. His studies grew out of the observation that the peasants of Europe used the macerated root of comfrey (*Symphytum officinale*) as an application for suppurative wounds, and the peasantry believed that the root of the comfrey had great healing power. Macalister made an extract of the comfrey roots and crystallized allantoin from the extract. He then tested the allantoin and demonstrated its healing properties.

Urea in the form of urine has been used as a healing agent by almost every primitive people. Lawrence of Arabia in his "Pillars of Wisdom" notes that it is generally used by the Bedouins. In the light of Robinson's studies there appears to be no question but that the primitive practices had merit. Today synthetic urea can be purchased at a negligible cost, and since the solutions are non-irritating to the most tender tissue and are non-toxic, it can be used without fear of untoward effects.

THE FORMATION OF CARBOHYDRATES AND FATS FROM AMINO ACIDS.—Carbohydrates and fats may be formed from proteins under the normal processes of metabolism. In studies of the factors influencing sugar excretion by diabetics, it had long been suspected that protein might give rise to carbohydrates.

Lusk administered amino acids to phlorizinized dogs and found that certain of the amino acids yielded sugar, whereas others did not. Those containing 2, 3, 4, and 5 carbon atoms in a straight chain were more or less completely converted into glucose. Glycine and alanine could be quantitatively converted into glucose, whereas only three of the carbon atoms of aspartic and glutamic acids were converted into glucose, three-fourths of the carbon of the aspartic acid and three-fifths of the carbon of the glutamic acid appearing in the form of glucose. Lusk believes that the intermediate products are either glycolic acid, lactic acid, or glyceric acid, depending on the amino acid involved. Thus three molecules of glycine on deamination would yield

[16] Macalister, C. J., A New Cell Proliferent: Its Chemical Application in the Treatment of Ulcers, *Brit. Med. J.*, (1912) 1: 10–12.

three molecules of glycolic acid which are reduced to three molecules of glycolic aldehyde, which in turn condense to one molecule of glucose.

$$\begin{array}{l}CH_2{-}NH_2\\ |\\ COOH\end{array} \longrightarrow \begin{array}{l}CH_2OH\\ |\\ COOH\end{array} \longrightarrow \begin{array}{l}CH_2OH\\ |\\ CHO\end{array} \longrightarrow C_6H_{12}O_6$$

And two molecules of alanine would yield two molecules of lactic acid which condense to form one molecule of glucose, or aspartic acid may yield β-lactic acid and carbon dioxide, two molecules of the lactic acid condensing to form glucose. Again glutamic acid may yield glyceric acid and acetic acid, the glyceric acid being transformed into glyceric aldehyde, two molecules of which condense to form one molecule of glucose.

Dakin has shown that serine, proline, arginine, and ornithine will yield at least a part of their carbon in the form of glucose, the arginine going first to ornithine. Lysine is the only straight-chain amino acid tested which failed to yield sugar. Apparently the aminopropionic acid side chain of tyrosine and phenylalanine is not available for glucogenesis, since glucose does not arise when these amino acids are fed.

The formation of fats from amino acids is a more difficult problem for investigation than the formation of sugars. There is no question, however, but that fat can be formed in the animal body from protein. We know that carbohydrates are readily converted into fats so that at least those amino acids which can give rise to glucose in the body can likewise give rise to fats. Possibly certain other of the amino acids may give rise to fats through side reactions. Thus leucine, tyrosine, and phenylalanine, though they do not directly give rise to glucose, do appear at least in part as acetoacetic acid, which should be convertible into fatty acids.

The Specific Dynamic Action of Proteins.—One additional property of proteins and amino acids in metabolism deserves mention, *i.e.*, the phenomenon which Rubner called the specific dynamic action of proteins. It had been noted by many workers that increased metabolism, as measured in a respiration calorimeter, resulted when proteins were fed. If an amount of sugar equivalent to a given number of calories is fed to a fasting dog, the heat, as measured in a respiration calorimeter, is slightly lower than the heat which would be produced by the combustion of the sugar which was fed. If, however, protein having an equivalent calorific value replaces the sugar, then the heat, as measured in a respiration calorimeter, exceeds by approximately 5 per cent the calorific value of the protein which was fed.

This phenomenon of excess heat production following the feeding of protein and of amino acids has been extensively investigated by Lusk and Benedict, who have shown that the excess heat is not due to mechanical heat from increased intestinal activity or peristaltic action.

Lusk has suggested that the amino acids behave as cell stimulants, raising the metabolic power of the tissue cells, the excess energy being derived from carbohydrates and fats which are burned in amounts greater than the normal level. He suggested that perhaps the stimulant is the keto or the oxy acids formed as intermediate metabolic products.

Borsook[17] has recently reviewed critically the literature on the specific dynamic action of protein and amino acids, and his paper may be consulted for the literature in the field. He concludes that the suggestion of Lusk of a cell stimulant is probably unnecessary and in any event contributes only a minor fraction to the excess calories. Borsook notes that there are two factors which account for the increase in metabolism after the ingestion of protein or amino acids. One of these factors is approximately constant and includes the increased oxygen consumption necessary for the oxidative deamination of the amino acids. One molecule of oxygen is used for each molecule of nitrogen deaminized. An increase in metabolism of 4 calories per gram of nitrogen, therefore, arises from oxidative deamination. In the synthesis of urea from ammonia and carbon dioxide we again have an evolution amounting to a maximum of 4 calories per gram of nitrogen. In order to excrete urea the kidney has to do work, and this work results in the production of heat. Borsook notes that this may amount to as much as 2 calories per gram of nitrogen. Thus, simply handling the nitrogen causes an increase in metabolism. Borsook's second source of increased metabolism concerns the utilization of the carbon residue after deamination. If the carbon residue is synthesized into glycogen, there will be an evolution of heat. He points out a number of possible and probable exothermic reactions which could adequately account for the increased metabolism which has been observed, and furthermore points out that the excess heat production which has been noted by various workers is not a constant but a variable and depends upon the conditions of the experiment and particularly upon the physiological state of the experimental animal.

Perhaps certain of the amino acids or their metabolic residues do act as cell stimulants. Tyrosine, when injected into the blood stream, causes a very appreciable increase in metabolic rate over that observed when even larger amounts of tyrosine are fed. Similarly the ingestion of 2-4-dinitrophenol has been found to have an enormous effect on metabolic rate. This compound, administered at a 20 mg. per kilo level, will increase the metabolic rate by as much as 1,000 per cent. The amino acid thyroxine, isolated by Kendall from the protein of the thyroid gland, apparently regulates in a large measure the metabolism of the body. The merest trace of thyroxine markedly raises

[17] Borsook, H., The Specific Dynamic Action of Protein and Amino Acids in Animals, *Biol. Rev.*, 11: 147–180 (1936).

the metabolic rate and maintains it at a high level for a considerable period of time. Perhaps other amino acids reflect in a small measure the extraordinary effects of thyroxine.

ENZYMATIC SYNTHESIS OF PROTEINS.—Bourquelot (*vide infra*) has shown that the reaction between glycosides and glycoside-splitting enzymes is a reversible reaction according to the following scheme:

$$\text{Glycoside} + \text{enzyme} \rightleftharpoons \text{enzyme} + \text{sugar} + x$$

where x represents a non-sugar radical. Accordingly, in dilute solutions the reaction is driven toward the right, resulting in the hydrolysis of the glycoside, whereas in concentrated solutions of the components plus the enzyme, the reaction is driven toward the left and the glycoside is resynthesized.

A number of workers [18] have attempted to synthesize proteins by means of a reversed enzyme reaction, adding the enzyme to a concentrated solution of protein split products. The only synthesis which is generally accepted is that by which Taylor appears to have synthesized the protamine *salmine*.

Taylor [19] digested the protamine, salmine, with trypsin, converted the amino acids into carbonates, destroyed the trypsin by heat, and concentrated the amino acids from 400 grams of the protamine to the point of crystallization. He then added 300 cc. of a glycerol extract from the liver of the clam *Schizothaerus nuttalii*, added toluene as a preservative, and set the mixture aside. The mixture gradually became opalescent, then cloudy, and finally a precipitate formed. At the end of 5 months, 2 grams of salmine sulfate was isolated from the mixture. A duplicate mixture, omitting the enzyme, yielded no trace of the protamine.

A very considerable number of workers have reported the synthesis of proteins particularly in the presence of pepsin. These proteins are generally referred to as *plasteins*. There appears to be no question but that an actual synthesis does take place. The only question involved is whether or not these proteins are analogous to the natural proteins. They are certainly much more complex than the proteose mixture from which they were derived. Wasteneys and Borsook believe that they do represent the products of a reversed equilibrium more or less similar to that noted above for the glycosides. However, they state that "peptic synthesis in concentrated peptic digests cannot be considered as an *in vitro* reproduction of biological processes; at best it is only a model." In this viewpoint Alcock agrees.

[18] For literature and general references see: Wasteneys, H., and Borsook, H., The Enzymatic Synthesis of Protein, *Physiol. Rev.*, 10: 110–145 (1930); and Alcock, R. S., *loc. cit.*

[19] Taylor, A. E., On the Synthesis of Protein through the Action of Trypsin, *J. Biol. Chem.*, 3: 87–94 (1907).

PROTEIN METABOLISM AND ORGANIC EVOLUTION.—This is the title of a paper by Joseph Needham.[20] The general thesis which is expounded is that the protein metabolism is a reflection of the conditions under which the embryos live, ammonia and urea being associated with an aquatic and uric acid with a terrestrial prenatal life. When the egg with a permeable membrane is laid in the water, the nitrogenous constituents, ammonia and urea, can readily be eliminated by diffusion. For terrestrial eggs, with no chance to get rid of nitrogenous excreta either through a semi-permeable membrane or by early hatching, some mechanism was necessary to prevent the accumulation of large quantities of a toxic product. This Needham believes was solved by the conversion of the nitrogenous wastes into uric acid and that this represented a definite step in the transition of animals from the sea to land.

[20] Needham, Joseph, Protein Metabolism and Organic Evolution, *Science Progress*, No. 92: 633–648 (1929).

CHAPTER XX

THE BIOLOGICAL REACTIONS OF THE PROTEINS

No discussion of the importance of the proteins in biological processes would be complete without at least a casual mention of the important role which they play in the problems of immunity. Wells,[1] Landsteiner,[2] and Marrack[3] have summarized the more important literature with particular reference to the chemical problems involved. Accordingly, in the following pages only a very brief outline of these questions will be considered.

Foreign proteins injected into a living animal, the injection being subcutaneous, intraperitoneal, or intravenous, give rise to the formation of specific substances in the blood serum of the animal, the presence of which may be detected by subsequent reactions of the animal or the blood serum of the animal. The reactions which follow the injection of foreign proteins may be classified into four groups (1) anaphylaxis, (2) the precipitin reaction, (3) hemolysis, and (4) complement fixation.

1. ANAPHYLAXIS.—When a small quantity of a protein (a *sensitizing* dose) is injected into the blood stream of an animal and an appropriate time interval (7 to 30 days or more, depending upon the initial dosage, a larger dosage requiring a longer time) is allowed to elapse, a second injection of the same protein (the *intoxicating* dose) will cause the animal to undergo a severe shock, death often ensuing within a few minutes. The physiological reaction which occurs is known as the anaphylactic shock. The amount of protein necessary for the sensitizing dose may be exceedingly small, as little as 0.000,000,05 gram of egg albumin being sufficient. The initial injection has sensitized the animal to this particular foreign protein. The second injection brings about the anaphylactic reaction.

2. THE PRECIPITIN REACTION.—When an initial injection of a foreign protein into an animal is followed by other injections at 3- to 4-day intervals, the dosage being gradually increased until an appre-

[1] Wells, H. Gideon, The Chemical Aspects of Immunity, Revised Edition, Chemical Catalog Company, New York (1929).

[2] Landsteiner, K., The Specificity of Serological Reactions, Charles C. Thomas, Springfield, Illinois, and Baltimore, Maryland (1936).

[3] Marrack, J. R., The Chemistry of Antigens and Antibodies, Special Rept. Ser., No. 194, Privy Council, Medical Research Council, H. M. Stationery Office, London (1934).

ciable quantity, 0.25 gram or more of the foreign protein, has been injected in a series of 5 to 6 up to 20 or 30 injections, the blood serum of the animal acquires the property of precipitating the particular foreign protein which was injected, when the immune serum is added to a solution of that protein *in vitro*. The delicacy of the test depends somewhat upon the nature of the protein injected and the potency of the immune sera used. The test is often used to detect human blood in criminal cases. Ordinary chemical tests for blood have a sensitivity which will detect dilutions of blood not much greater than 1 : 1000. The precipitin reaction has been found to be positive at a dilution of 1 : 50,000 for blood, and at a dilution of 1 : 1,000,000 for egg albumin. The "agglutination" reactions of bacteria are probably due to the precipitin reaction of proteins which are on the surface of the bacteria.

3. HEMOLYSIS.—When red blood cells of an animal are injected into the blood stream of an animal of a different species through a series of rather shortly spaced injections, the blood serum of the injected animal acquires the ability to dissolve the foreign blood corpuscles when tests are made *in vitro*. Here, again, the reaction has been employed in criminal cases to ascertain whether or not the cells in a blood stain were human corpuscles. Similarly, "bacteriolysins" may be formed, which will disintegrate specific bacteria. Natural or acquired immunity may at least in part be due to the presence of such bacteriolysins.

4. COMPLEMENT FIXATION.—This reaction depends upon the fact that immune sera contain at least two distinct substances, both of which are required for the production of an immuno reaction. These are (*a*) the relatively heat-stable "antibody," and (*b*) the complement which is destroyed at 55° C. Complement occurs in fresh, normal serum and can be supplied from that source.

Five biological reagents are necessary to carry out the complement fixation test.

Reagent 1 is a suspension of red blood cells (*e.g.*, sheep cells).

Reagent 2 is the immune serum A (*e.g.*, the serum of a patient who is suspected of having typhoid fever). The complement of this immune serum has been previously destroyed by heating the serum to 55° C.

Reagent 3 is a supply of fresh, normal serum to act as a source of complement.

Reagent 4 is the hemolytic serum B (*e.g.*, serum from rabbits which have been immunized to sheep corpuscles). This hemolytic serum B has had the complement destroyed by heating to 55° C.

Reagent 5 is a suspension of the bacteria causing typhoid fever.

The test is carried out by taking the immune serum A (reagent 2), adding complement (reagent 3), and then adding the bacteria suspension (*the antigen,* reagent 5). If reagent 2 is in reality a typhoid im-

mune serum, combination will occur between the typhoid bacteria, the typhoid antibody, and the complement (reaction I), which will remove all the complement from the solution and "fix" (adsorb ?) it on the agglutinated or precipitated bacteria. However, it may not be possible for us to detect this reaction. It is necessary, accordingly, to determine whether or not reaction I has taken place. It is here that reagents 1 and 4 are used. To the original mixture of typhoid bacteria, typhoid antibody and complement are now added reagents 1 and 4. *If hemolysis occurs, reaction I did not take place,* and complement is still present in the solution, as evidenced by the fact that the red cells were broken down by the hemolytic serum B. *If hemolysis does not take place, reaction I has already occurred, and reagent 2 was in reality serum from a patient with typhoid fever,* all the complement having been used up in reaction I. A modification of this reaction, but essentially the same so far as the technic is concerned, is the Wassermann test for syphilis.

HAPTENS.—Landsteiner[4] coined the name hapten, about 1921, to designate *substances which when injected alone do not give rise to the formation of antibodies in the blood serum but which, when combined with a protein and injected, give rise to immune sera specific for the chemical grouping of the compound (non-protein) which was present in the protein-hapten combination. In many instances the uncombined hapten reacts in vitro with the antibody so formed.* He accordingly notes that there are two systems of species specificity in the animal kingdom, the specificity of the proteins and the specificity of non-protein residues which may react as haptens, and that these specific non-protein substances may occur in the alcoholic extracts of cells and tissues, giving rise to antibodies. In the bacteria, in particular, *specific polysaccharides* (*vide infra*) have been isolated which are characteristic of particular bacterial types. These specific polysaccharides react as haptens, inasmuch as they do not, when injected in the pure state, give rise to the formation of antibodies but do *in vitro* react with specific immune serum characteristic of the particular type of bacteria containing the specific polysaccharide.

Our knowledge of the chemistry of immunity has had an amazing development during the last ten years. This development has been largely, but not exclusively, due to the work of Landsteiner and his co-workers and the work of Michael Heidelberger and his co-workers, although many others have been and are now active in the field. This development has been brought about by the demonstrated formation of specific antibodies reacting with specific haptens. The antibody formation was induced by combining a chemical compound possessing a definitely known structure with a protein and then injecting this de-

[4] Landsteiner, K., Über heterogenetisches Antigen und Hapten. XV. Mitteilung über Antigene, *Biochem. Z.*, 119: 294–306 (1921).

rived protein into the experimental animal. *The immune serum so produced is specific, by and large (vide infra), for the specific chemical grouping of the non-protein constituent combined in the derived protein.* The developments in this field are covered by Chapter V of Landsteiner and Chapter III of Marrack, and the student interested in biological reactions is urged to read these chapters.

The original observations that non-protein constituents may give rise to antibody formation may be traced back to Obermayer and Pick,[5] but it was not until the observations of Pauly,[6] followed by the work of Landsteiner and Prasek and Landsteiner and Lampl,[7] that the phenomenon was recognized as general. In these later studies *acyl* groups were introduced into the protein and the immune sera were found to react with acyl-substituted proteins, being more or less specific for the particular acyl group which had been introduced and relatively non-specific for the original protein. The next advance was the formation of *azoproteins*, where proteins were coupled with a diazonium compound to form azo derivatives. *These azoproteins gave rise to immune sera specific for the diazonium group which had been introduced into the protein,* so that it became possible to study immuno reactions characteristic of particular and of known chemical groupings. Conclusive proof has developed that the diazonium compounds couple with the tyrosine and histidine residues in the protein and that the immuno reactions of the resulting azoprotein reflect the specific configuration of the chemical residues linked to the azo-group. Table LXVI shows the cross-reactions of immune sera for a series of azoproteins differing somewhat in chemical constitution with a series of antigens consisting of azoproteins containing different hapten residues. It will be noted that the immune serum for the azoprotein containing *p*-aminobenzoic acid as a hapten reacted strongly with its own azoprotein, very weakly with an azoprotein containing *m*-aminobenzoic acid, but that there was no reaction with *o*-aminobenzoic acid or with a considerable number of closely related compounds even in-

[5] Obermayer, F., and Pick, E. P., Vorläufige Mitteilung, *Wien klin. Wochschr.*, 16: 659–660 (1903); Beiträge zur Kenntnis der Präzipitinbildung, *ibid.*, 17: 265–267 (1904); and Über die chemischen Grundlagen der Arteigenschaften der Eiweisskörper, *ibid.*, 19: 327–334 (1906).

[6] Pauly, H., Über die Konstitution des Histidins, I., *Z. physiol. Chem.*, 42: 508–518 (1904); Zur Kenntnis der Diazoreaktion des Eiweisses, *ibid.*, 94: 284–290 (1915).

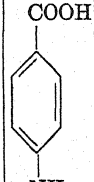
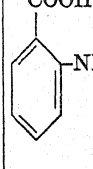
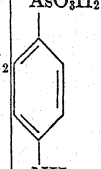
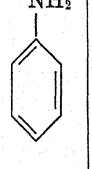
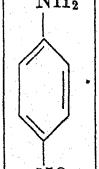
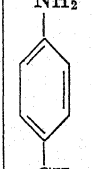
[7] Landsteiner, K., and Prasek, E., Notiz zu der Mitteilung über Immunisierungsversuche mit Lipoproteinen, *Biochem. Z.*, 61: 191–192 (1914); and Landsteiner, K., and Lampl, H., Über Antigene mit verschiedenartigen Acylgruppen, X, *Z. Immunitäts.*, 26: 258–276 (1917); Über die Antigeneigenschaften von Azoproteinen, XI, *ibid.*, 26: 293–304 (1917); Über die Abhängigkeit der serologischen Spezifizität von der chemischen Struktur (Darstellung von Antigenen mit bekannter chemischer Konstitution der spezifischen Gruppen. XII. Mitteilung über Antigene, *Biochem. Z.*, 86: 343–394 (1918).

cluding *p*-aminophenylarsenic acid. Similarly when *m*-aminobenzoic acid is diazotized and linked to protein, and this protein injected into an animal to form an immune serum, the immune serum will react with

TABLE LXVI

SHOWING CROSS-REACTIONS BETWEEN IMMUNE SERA FOR AZOPROTEINS CONTAINING CERTAIN GROUPINGS AND AZOPROTEIN ANTIGENS CONTAINING VARIOUS HAPTENS

[LANDSTEINER AND VAN DER SCHEER, *J. Exptl. Med.*, 45: 1045–1056 (1927)]

Azoprotein Antigen Containing the Residue from Diazotized	Immune Sera for Azoprotein Containing the Residue from Diazotized					
						
p-Aminobenzoic acid...	+++	0	0	0	0	0
m-Aminobenzoic acid..	±	0	0	0	0	0
o-Aminobenzoic acid...	0	++	0	0	0	0
p-Aminophenylarsenic acid.....	0	0	++++	0	0	0
Sulfanilic acid.....	0	0	0	0	0	0
o-Aminocinnamic acid..	0	0	0	0	0	0
Aniline.....	0	±	0	++±	+	++
p-Nitroaniline.....	0	±	0	+	++	+±
p-Toluidine.....	0	....	....	+±	+	++±
m-Toluidine.....	0	±	±	++	+	+±

the azoprotein which was initially injected but will not react with other azoproteins derived by the coupling of the diazonium salts of the following compounds with protein: aniline, *o*-aminobenzoic acid, amino-*m*-toluic acid, amino-*o*-toluic acid, 3-amino-4-methylbenzoic acid, 4-chloro-3-aminobenzoic acid, 4-bromo-3-aminobenzoic acid, *p*-aminobenzoic acid, *o*-aminocinnamic acid, *m*-aminocinnamic acid, *p*-aminocinnamic acid, *o*-aminobenzene sulfonic acid, 4-bromoaniline-2-sulfonic acid, *m*-aminobenzene sulfonic acid, and 4-aminotoluene-2-sulfonic acid. Thus, the introduced group induces a remarkable specificity.

Perhaps a still more pronounced illustration of the specificity of the induced hapten group occurs in the experiments of Landsteiner and

van der Scheer,[8] where *d*-, *l*-, and *meso*-tartaric acids were introduced into proteins. These data are shown in Table LXVII. It will be

TABLE LXVII

SHOWING THE REACTIONS OF IMMUNE SERA (WHERE *d*-, *l*-, AND *meso*-TARTRANILIC ACIDS WERE INTRODUCED INTO THE IMMUNIZING AZOPROTEIN) WITH THE CORRESPONDING PROTEIN-HAPTEN ANTIGENS. A DEMONSTRATION OF THE EFFECT OF STEREOISOMERIC CONFIGURATION ON IMMUNOSPECIFICITY. (ANTIGEN CONCENTRATION: FIRST COLUMN = 0.05%, SECOND COLUMN = 0.01%.) (FROM LANDSTEINER, *loc. cit.*, BY PERMISSION. COURTESY CHAS. C. THOMAS, PUBLISHERS.)

Immune Sera from	Antigen from					
	l-Tartaric Acid		*d*-Tartaric Acid		*meso*-Tartaric Acid	
l-Tartaric Acid....	+++	++±	±	0	+	±
d-Tartaric Acid....	0	0	+++	++±	+	±
meso-Tartaric Acid	±	±	0	0	+++	+++

noted that there is a certain cross-reaction between the sera from the active forms of the tartaric acids and those from the *meso* form. This Landsteiner explains by the fact that the *meso* form contains carbon atoms having both *d*- and *l*-configuration, and accordingly, while the reaction is not as intense with the *meso* form, nevertheless it does indicate similarity of chemical configuration. From these and other observations in the literature, it becomes evident that *the stereoisomeric configuration around an asymmetric carbon atom can be detected by serological reactions.* A β-glucoside hapten and a β-galactose hapten are immunologically distinct. Even α- and β-glucoside haptens, when linked in a protein, can be distinguished from each other serologically. The serological differentiation appears to be as pronounced as the corresponding α- and β-glycosidal enzyme relationships which have been known for so long. When polypeptides are used as haptens and introduced into protein residues, Landsteiner found that "the specificity is determined chiefly by the amino acid carrying the free carboxyl group, to a lesser degree by the second amino acid" from the carboxyl end of the polypeptide chain. Landsteiner notes that those haptens which contain a free carboxyl, sulfonic or arsenic acid grouping give rise to a much greater antigen specificity than those in which the substituting groups are neutral. Apparently an acidic residue increases the specificity of the reaction.

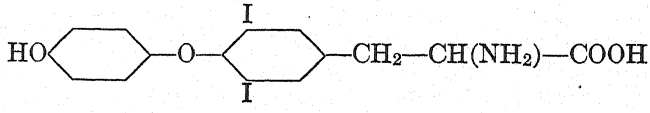
[8] Landsteiner, K., and van der Scheer, J., Serological Differentiation of Steric Isomers (Antigens Containing Tartaric Acids), *J. Exptl. Med.*, 50: 407–417 (1929).

We have already noted that the original biological specificity of the protein into which the hapten was introduced is, to a very considerable extent, lost and that the hapten-protein compound acquires a new specificity. In his discussion of this question Landsteiner points out that in many instances the orginal specificity of the protein is retained to some extent and can be detected by very careful technics, but that the specificity for the introduced hapten in general overshadows in intensity the original specificity of the protein. Hopkins and Wormall,[9] using phenylisocyanate or bromphenylisocyanate to form the phenylureidoprotein derivatives, found that the original protein specificity was lost and that the immune sera from the ureido derivatives would interact with all sorts of ureidoproteins including even gelatin. Similarly xanthoproteins lose their protein specificity, and the immune serum for one xanthoprotein will react with other nitrated proteins regardless of whether they are derived from plant or animal sources. Halogenated proteins similarly give rise to immune sera specific for halogenated proteins. The iodoprotein specificity apparently resides in the 3.5-diiodotyrosine residue, so that any protein containing this residue would react with the immune serum from any other protein containing that residue. Methylated and acetylated proteins also lose part or all of the original protein specificity and acquire new specificities for the introduced groups.

We have already noted that simple haptens do not form precipitates with the corresponding immune sera *in vitro* but that precipitates are formed only with the immune sera and the protein-hapten complex. Landsteiner, however, observed that, *if the immune serum was mixed with the simple hapten and then mixed with the protein coupled with the hapten, no precipitate formed.* He designated this as *the phenomenon of specific inhibition,* and an enormous literature is being built up in this field. It is a phenomenon very similar and probably closely related to the phenomenon of complement fixation. Apparently the soluble hapten couples with or neutralizes the immune serum, but since no precipitate forms, there is no visible evidence of the reaction or coupling since the resulting product is still soluble. However, the original coupling with the hapten prevents a later coupling with the protein-hapten complex. This allows for a study (*in vitro*) of the serum reactions of simple substances of known constitution which are themselves devoid of antigenic power but which can combine specifically with antibodies. In the case of the halogenated proteins where 3.5-dihalogenated tyrosine is the specific hapten, it has been

[9] Hopkins, S. J., and Wormall, A., Phenyl *Iso*cyanate Protein Compounds and Their Immunological Properties, *Biochem. J.*, 27: 740–753 (1933); II. The Gelatin Compounds, *ibid.*, 1706–1715 (1933); III. The Amino-Acid Derivatives and Serological Inhibition Tests, *ibid.*, 28: 228–236 (1934).

shown that thyroxine is effective as a specific inhibitor but that diiodothyronine,



does not inhibit. Apparently the two iodines must be *ortho* to the —OH group. In the case of the phenylureido compounds, the ϵ-amino group of lysine was found to be the point of attachment for the grouping, for phenylureidolysine acted as a hapten in the specific inhibition reaction of ureidoproteins.

Landsteiner goes further and suggests that hay fever, allergies of various kinds, and acquired hypersensitivity to chemicals which is often observed in the manufacture of dyestuffs, etc., as well as hypersensitivity to formaldehyde and to specific drugs, may in many instances be due to compounds of small molecular weight becoming in some way attached to a protein molecule and thus having induced the initial sensitization of the individual. In his experiments, in certain instances when the animal has been sensitized to a protein coupled with a hapten it has been possible to induce an anaphylactic shock by an appropriate dosage of the pure uncoupled hapten. It seems as though any compound containing labile hydrogen, halogen, or other labile atomic groupings might, if it secures entrance to the blood stream, spontaneously couple with some of the serum proteins, thus giving rise to sensitization. In certain systems the specific inhibition reaction has been applied to the serum of hypersensitive individuals, and haptens of low molecular weight have been detected as being responsible for the hypersensitivity.

There is some evidence that even coupling with a protein may not be necessary, for workers have reported the production of antibodies where the hapten was adsorbed on hydrous aluminum oxide or on charcoal. It may be that the compound circulating in the blood stream must either be in the colloidal state or have its kinetic energy greatly reduced. It seems possible that a reduction in kinetic energy might be advantageous in inducing specific orientation on the surface of tissue cells or possibly ingestion by phagocytes or tissue cells as a preliminary to antibody formation.

The Nature of the Antibody-Antigen Combination.—Marrack reaches the conclusion that the combination of antibody with antigen is an adsorption reaction which is fitted by the Freundlich adsorption isotherm. When small amounts of antibody are present practically all the antibody is fixed by the antigen. With increasing amounts of antibody, holding the antigen constant, a part of the antibody appar-

ently remains uncombined, the amount remaining uncombined being greater as the concentration of the antibody increases. If the logarithms of the amount bound are plotted against the logarithms of the amount remaining free, a straight line results, as would be anticipated from an adsorption isotherm.

Hydrogen-ion concentration has a marked effect on antibody-antigen combination. In one case which Marrack cites, combination was constant and maximum in the range of *p*H 5.55 to *p*H 8.0. The combination was markedly decreased and the reaction was much slower at *p*H 4.5 and at *p*H 9.5, and was completely inhibited at a *p*H 3.0 or less or *p*H 10.0 or greater.

Salt effects are likewise prominent as affecting the antibody-antigen combination. The anions show specific lyotropic series effects either in inhibiting the combination or in dispersing the precipitate which forms, with CNS^- standing at the dispersing end of the series and F^- at the coagulating end. Marrack further suggests that a secondary reaction more or less similar to the heat denaturation of proteins may follow the primary adsorption reaction.

ARE THE IMMUNO REACTIONS SPECIFIC FOR A PARTICULAR PROTEIN? —For a number of years immunologists have been in general agreement that *protein specificity depends upon the chemical structure of the protein molecule and not necessarily upon the biological origin of the protein,* although the great majority of proteins of different species are different both chemically and immunologically. In certain instances, however, immunological identity, or at least close similarity, has been observed in proteins isolated from different biological sources. Thus, the globulin of the squash seed appears to be immunologically identical with the globulin from the seeds of the cantaloupe, and there is an immunological similarity between ovomucoid from hens' eggs and a mucoid-like protein present in the seeds of the sweet almond. The caseins from the milk of animals of different species show very close biological relationships. The same is true of the vitellins of the egg yolk from various species of animals. Wells notes that "casein from the milk of an animal of any given species shows a closer biologic relationship to the casein of another species than it does to either the whey proteins or the serum proteins of its own species," and "egg yolk proteins from even such widely different species as fish and turtle may give precipitin reactions with the antiserum for hen egg yolk proteins."

Such of the keratins as have been studied appear to show very little species specificity, and the lens protein [10] of the eye of one animal reacts with the lens protein from the most diverse species of animals.

[10] Uhlenhuth, Festschrift, Robert Koch, Jena (1903) (cited by Landsteiner, *loc cit.*); Hoffmann, H., Experimentelle Untersuchungen über die Fraktionen des Linseneiweisses, *Z. Immunitäts.*, 71: 171–187 (1931).

From the observation of proteins deliberately coupled with haptens, it has been absolutely demonstrated that immuno reactions are specific for particular chemical groupings, and that substances containing very closely related, but nevertheless slightly different, chemical groupings will react with the immune bodies to a greater or less extent. As the degree of chemical relationship diverges, there is a lessening in the intensity of the serological reaction until finally the serological reaction is undetectable. Similarly in proteins the species specificity is not absolute but varies with the biological relationships of the organisms. Closely related species apparently have proteins which are not absolutely identical but chemically closely related. As the degree of relationship becomes less, the chemical differentiation of the protein becomes greater until finally there are no cross-reactions between the immune sera. Occasionally it may happen that an organism synthesizes proteins which contain chemical groupings similar to or identical with chemical groupings in a protein synthesized by an organism in a different biological species or genera. In such cases cross immunological reactions should not be interpreted to indicate phylogenetic relationships but rather identity or similarity of chemical groupings. Manwaring [11] points out that proteins may be 99 per cent identical but that the immunological tests may be measuring the 1 per cent which is non-identical, and that the immuno reactions are for specific groupings rather than for the entire protein molecule.

PHYLOGENETIC RELATIONSHIPS AS DETERMINED BY SEROLOGICAL REACTIONS.—A number of workers have used serological studies to trace phylogenetic relationships in both the plant and the animal kingdoms. Nuttall [12] studied more than 16,000 precipitin reactions quantitatively with the blood of more than 900 species of animals and found that antihuman precipitating serum gave comparative volumes of precipitate when tested against the blood sera of various orders of primates, as shown in Table LXVIII. Landsteiner and Miller [13] have added to these observations.

Martin and Cotner [14] studied the reaction of fourteen genera and twenty species of moths in six subfamilies of the *Phalaenidae* and concluded that the serological reactions were very useful in the determina-

[11] Manwaring, W. H., Post Ehrlich Immunology, *Science*, 74: 324–327 (1931), *cf.* also Biochemical Relativity, *Science*, 72: 23–27 (1930).

[12] Nuttall, G. H. F., Blood Immunity and Blood Relationship, Cambridge University Press (1904).

[13] Landsteiner, K., and Miller, C. P., Jr., Serological Observations on the Relationship of the Bloods of Man and the Anthropoid Apes, *Science*, 61: 492–493 (1925).

[14] Martin, S., and Cotner, F. B., Serological Studies of Moth Proteins with Special Reference to Their Phylogenetic Significance, *Ann. Entomol. Soc. Am.*, 27: 372–383 (1934).

TABLE LXVIII

THE INTENSITY OF THE PRECIPITIN REACTION BETWEEN THE SERUM OF AN ANIMAL IMMUNIZED AGAINST HUMAN BLOOD PROTEINS AND EQUIVALENT AMOUNTS OF BLOOD SERA FROM VARIOUS ORDERS OF PRIMATES.

Blood Serum from	Number of Individuals Tested	Intensity * of Precipitin Reaction
Human	34	100
Simiidae (anthropoids)	8 (3 species)	100
Cercopithecidae (common monkeys of the old world)	36	92
Cebidae (capuchins and spider monkeys of the new world)	13	78
Hapalidae (marmosets)	4	50
Lemuridae (lemurs)	2	0

* 100 indicates a reaction essentially identical in intensity with that of the original serum used for immunizing.

tion of phylogenetic relationships. Irwin and Cole,[15] in studying the immunogenetic relationships of the erythrocytes of the common pigeon and doves and hybrids between the various species, find that the

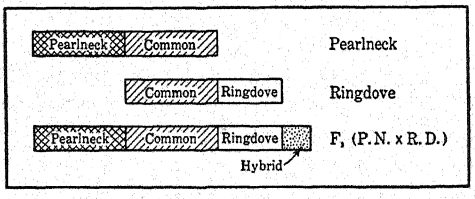


FIG. 138.—Showing diagrammatically the immunologically specific components of the erythrocytes of "Pearlneck" and "Ring dove" and the F_1 hybrids. (After Irwin and Cole.)

erythrocytes possess many substances in common but that *each species possesses specific immunodetectable compounds peculiar to the species* and that these can be traced through the F_1 hybrids and that they segregate in the backcross generations. Thus, there are species specific

[15] Irwin, M. R., and Cole, L. J., Dissimilarities Between Antigenic Properties of Red Blood Cells of Dove Hybrid and Parental Genera, *Proc. Soc. Exptl. Biol. Med.*, 29: 850–851 (1932); Immunogenetic Studies of Species and of Species Hybrids in Doves, and the Separation of Species-Specific Substances in the Backcross, *J. Exptl. Zool.*, 73: 85–108 (1936); Immunogenetic Studies of Species and of Species Hybrids in Pigeons, and the Separation of Species-Specific Characters in Backcross Generations, *ibid.*, 73: 285–308 (1936); Immunogenetic Studies of Species and of Species Hybrids from the Cross of *Columba livia* and *Streptopelia risoria*, *ibid.*, 73:309–318 (1936).

"Pearlneck" substances, species specific "Ring dove" substances, etc. Figure 138 shows this diagrammatically for the Pearlneck dove, Ring dove, and the F_1 hybrid. In the F_1 hybrid a new immunologically specific "hybrid" substance likewise appeared. The chemical nature of these species specific substances has not been ascertained.

In the plant kingdom we have the studies of Magnus,[16] Zade,[17] Mez,[18] and Moritz.[19] Certain of these studies have dealt with the genetic relationships of the *Leguminosae* and the *Gramineae*. Others, particularly those of Mez and Moritz, have been directed primarily toward phylogenetic problems. Mez has surveyed almost the entire plant kingdom and constructed a chart showing phylogenetic relationships of the plant kingdom based upon serological reactions. This chart is reproduced in Fig. 139.

The phylogenetic relationships shown in this chart are in most instances similar to or identical with those which have been generally accepted by taxonomists. In some instances the serum-diagnostic method of Mez indicates relationships different from those usually accepted. The author, however, has discussed this chart with several taxonomic botanists and in each instance has received the assurance that the chart may well express the true phylogenetic relationships. It would accordingly seem as if serum diagnosis may be used in questions of disputed phylogeny. Moritz in his studies has extended and confirmed the observations of Mez and concludes that the technics which Mez used were adequate.

We have already noted that Lewis and Wells found immunological relationships between the prolamines of *Triticum vulgare, T. dicoccum, T. monococcum,* and *T. spelta.* They similarly found immunological relationships between the prolamines of the corn group, *Zea mays,*

[16] Magnus, W., Die Erkennung von Mehlverfälschungen durch die serumdiagnostische Methode, *Landw. Jahrb.*, 38 (Erg. Bd. V): 207–215 (1909); Weitere Ergebnisse der Serum-Diagnostik für die theoretische und angewandte Botanik, *Ber. Botan. Ges.*, 26a: 532–539 (1908).

[17] Zade, A., Serologische Studien an Leguminosen und Gramineen, *Z. Pflanzensüchtung*, 2: 101–151 (1914); Die Verwendbarkeit der Präzipitinreaktion in der Samenprüfung, *Fuhlingslandw. Ztg.*, 61: 807–810 (1912).

[18] Mez, C., and Ziegenspeck, H., Zur Theorie der Sero-Diagnostik, *Botan. Arch.*, 12: 163–202 (1925); *cf.* also numerous papers published in *Botanisches Archiv* under the general title, "Sero-diagnostische Untersuchungen," and Mez, C., Sero-Diagnostic Methods Applied to Drug Examination, *Proc. Intern. Congr. Plant Sci.*, pp. 1402–1405 (1926).

[19] Moritz, O., Zur Kritik der Phytoserologie, *Biol. Zentr.*, 48: 431–443 (1928); Betractungen zum "Ende" der botanischen Serodiagnostik, *Beih. Botan. Centralbl.*, 46: 114–118 (1929); Weitere Beiträge zur Kritik und zum Ausbau phytoserologischer Methodik, *Planta*, 7: 759–814 (1929); Serologische Studien über das Linswickenproblem, *Biol. Zentr.*, 51: 290–307 (1931); Prinzipien und Beispiele der Anwendung phytoserologischer Methodik, *Planta*, 15: 647–696 (1932); Serologische Untersuchungen an Getreidebastarden, *Ber. deut. botan. Gesell.*, 51: 52–57 (1933); Die botanische Serologie, *Beitr. Biol. Pflanz.*, 22: 51–90 (1934).

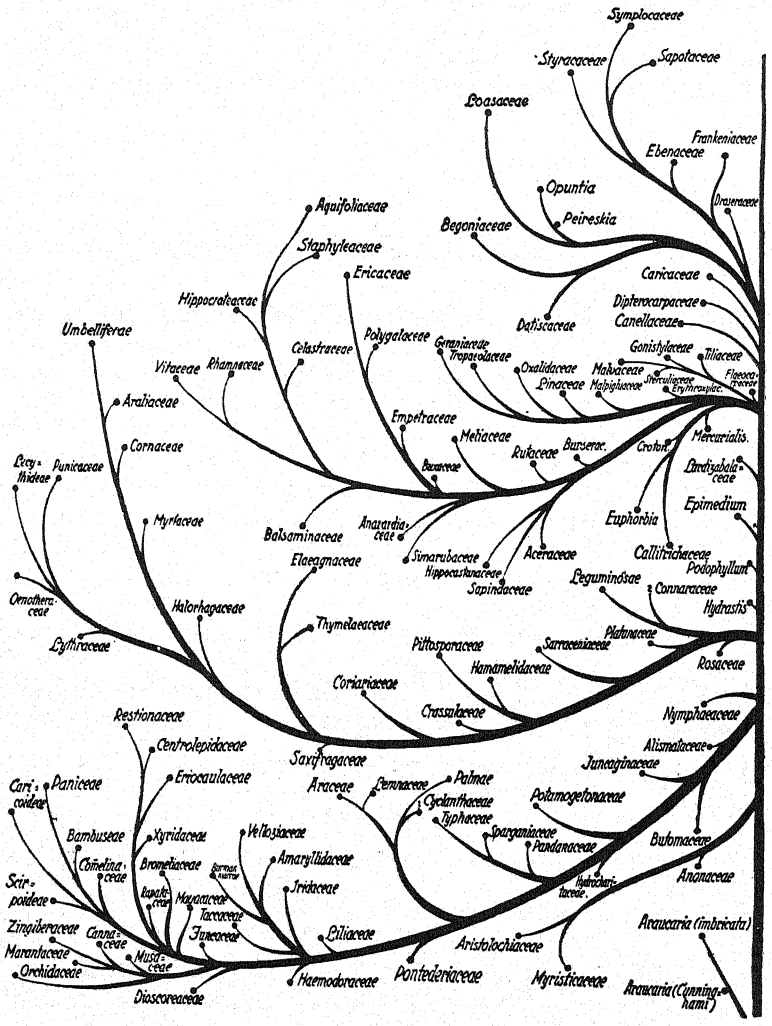


FIG. 139A.—The phylogenetic relations of plant species and genera, as indicated by serologic reactions. (Data of Mez.) Reproduced by permission of Dr. Carl Mez and the *Botanisches Archiv*.

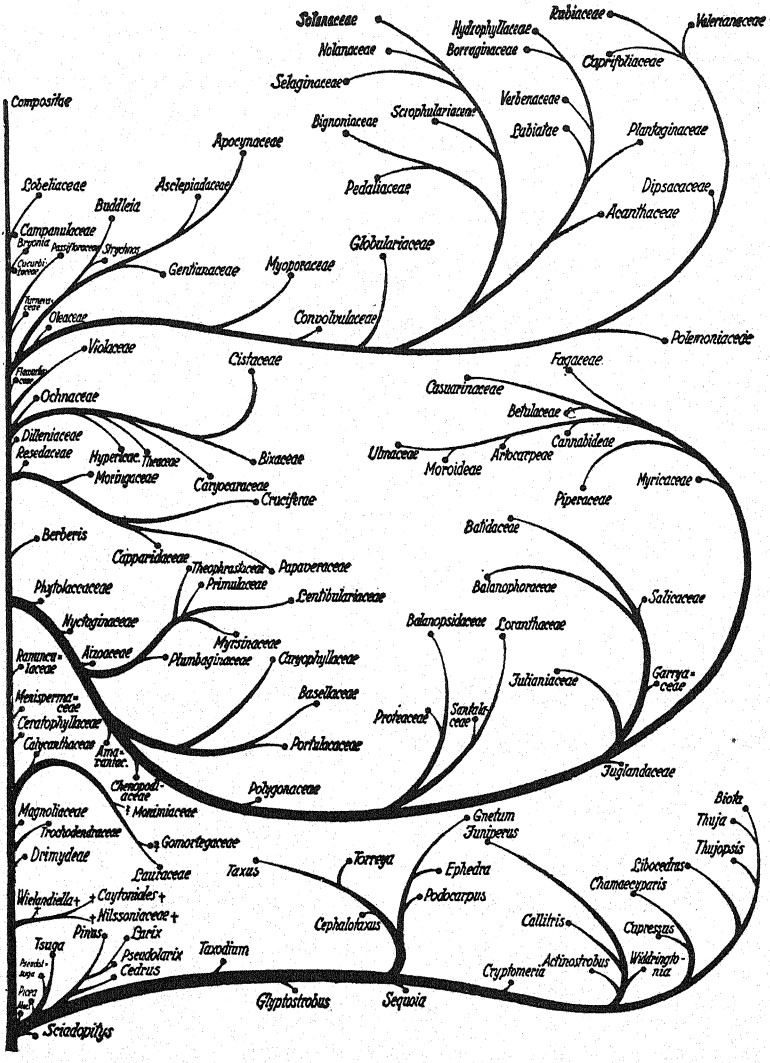


FIG. 139B.—The phylogenetic relations of plant species and genera, as indicated by serologic reactions. (Data of Mez.) Reproduced by permission of Dr. Carl Mez and the *Botanisches Archiv*.

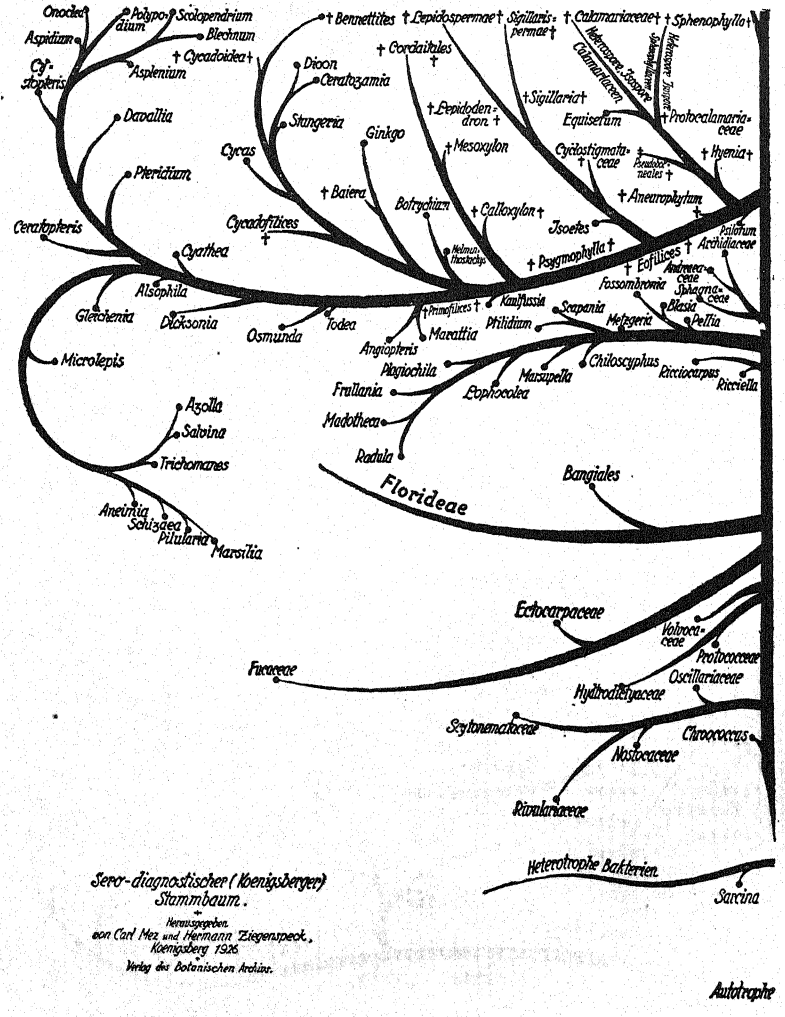


FIG. 139C.—The phylogenetic relations of plant species and genera, as indicated by serologic reactions. (Data of Mez.) Reproduced by permission of Dr. Carl Mez and the *Botanisches Archiv*.

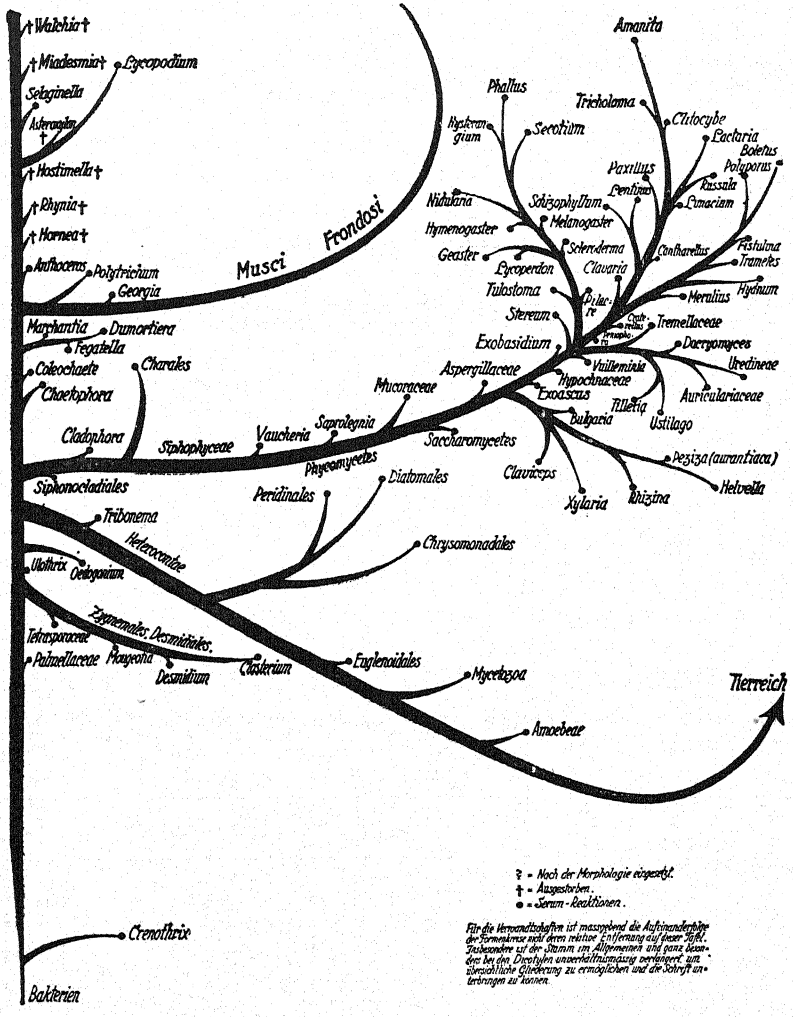


FIG. 139D.—The phylogenetic relations of plant species and genera, as indicated by serologic reactions. (Data of Mez.) Reproduced by permission of Dr. Carl Mez and the *Botanisches Archiv*.

Andropogon sorghum, and *Euchlaena mexicana* Schrad, but no relationship between the "wheat" group and the "corn" group.

In a later study[20] the globulins from the Georgia velvet bean and the Adzuki, Mung, navy, and lima beans were compared immunologically. The Georgia velvet bean globulins were distinct from those of the four other varieties. The lima and navy bean globulins were more or less alike but distinguishable from each other. The globulins from the Adzuki and Mung beans were similar or closely alike but were apparently not closely related to those of the other globulins tested. The Georgia velvet bean belongs to the genus *Stizolobia,* all the others to the genus *Phaseolus.*

Blood Groups.—Normal sera of the higher animals usually contain substances which agglutinate or hemolyze the red cells of individuals of the same species. These have been designated as isoagglutinins or isohemolysins. The presence of isoagglutinins or isohemolysins in human blood, together with the corresponding antigens in blood cells, accounts for the four blood groups in man which were discovered by Landsteiner,[21] which together with his later studies in the field of immunology earned for him the award of the Nobel prize in 1930. In man there are two isoagglutinins and two agglutinable substances. The α- and β-isoagglutinins are inherited in the Mendelian fashion as genetic dominants. Table LXIX shows the blood groups in man, the isoagglutinins in the serum, and the reaction with erythrocytes from the various groups. The anthropoid apes contain

TABLE LXIX

Immuno Reactions Characteristic of the Four Blood Groups in Man. (From Landsteiner, *loc. cit.*, by Permission. Courtesy Chas. C. Thomas, Publisher.)

Blood Group	Isoagglutinins in Serum	Reactions with Erythrocytes of Group			
		O	A	B	AB
O	α and β	0	+	+	+
A	β	0	0	+	+
B	α	0	+	0	+
AB	...	0	0	0	0

[20] Wells, H. Gideon, Lewis, J. H., and Jones, D. Breese, Immunologic Reactions of the Globulins from the Seeds of Leguminous Plants, *J. Infectious Diseases,* 40: 326–342 (1927).

[21] Landsteiner, K., Zur Kenntnis der antifermentativen, lytischen und agglutinierenden Wirkungen des Blutserums und der Lymphe, *Centr. Bak. Parasitenk. Infek.,* 27: 361–362 (1900); *cf.* also, Individual Differences in Human Blood, *Science,* 73: 403–409 (1931).

isoagglutinins and agglutinable substances indistinguishable from those in human blood. Chimpanzees, in contrast to the four groups in man, have only two blood groups corresponding to the O and A groups in man.

Racial differences in man are markedly shown by differences in blood groupings. This was first demonstrated by the Hirszfelds.[22] Only group O occurs in the full-blooded American Indian. Agglutinable substance A is much more common than agglutinable substance B in Europeans and Australian blacks. In the Hindus the ratio is reversed (Englishmen 46.4 per cent A, 10.2 per cent B; Hindus 49.7 per cent B, 27.5 per cent A). Blood grouping has even been used to trace race relationships in Egyptian mummies and likewise in cases of disputed parentage. Races of animals show distinctive blood groupings within the species.

Chemically Induced and Directed Genetic Transformations.—It has been the dream of the geneticist for years to acquire the technic of inducing by experimental technics changes in an organism which are heritable and which will be passed on to the offspring. Isolated reports of success which have appeared from time to time have in general failed to be confirmed. Therefore, the experiments of Dawson and Sia,[23] and of Alloway [24] take on added significance, inasmuch as these workers not only have apparently effected the permanent transformation of one type of organism into another heritable type of organism, but they have been *directed* changes in which the conditions of the experiment determine the direction which the inheritable modification will take, and in addition cause the production within the new type of organism of specific chemical compounds chemically and immunologically distinct from the chemical compounds which the original organism could elaborate. In Alloway's experiments in particular this was accomplished by the growing of pneumococci of one type in a culture medium to which had been added a sterile filtered extract of a different type organism. Thus, culture of Type R Pneumococci were grown in culture media to which were added extracts of Type I-S, Type II-S, and Type III-S Pneumococci and under such conditions *the type R Pneumococci changed to the particular type characteristic of the material from which the active extract was derived.* The active

[22] Hirszfeld, L., and Hirszfeld, H., Serologische Untersuchungen bei den Rassen der Menschen, *Przeglad epidemjol*, 1 (Heft 2): 1–13 (1920).

[23] Dawson, M. H., and Sia, R. H. P., *In Vitro* Transformation of Pneumococcal Types. I. A Technique for Inducing Transformation of Pneumococcal Types *in Vitro*, *J. Exptl. Med.*, 54: 681–699 (1931); II. The Nature of the Factor Responsible for the Transformation of Pneumococcal Types, *ibid.*, 54: 701–710 (1931).

[24] Alloway, J. L., The Transformation *in Vitro* of R Pneumococci into S Forms of Different Specific Types by the Use of Filtered Pneumococcus Extracts, *J. Exptl. Med.*, 55: 91–99 (1932); Further Observations on the Use of Pneumococcus Extracts in Effecting Transformation of Type *in Vitro*, *ibid.*, 57: 265–278 (1933).

extract could be clarified with charcoal and when so clarified was water-clear, yet the filtrate contained practically all the activity. The active substance could be precipitated from this filtrate with alcohol of 70–100 per cent concentration or with acetone. No loss in potency resulted from precipitation. Filtrates from the precipitate were inactive. The potent factor was thermolabile but was occasionally active after 10 minutes' exposure to 90° C. The extract passed through both N and W types of Berkefeld filters and retained its activity. The action of the extracts was specific. An extract from Type II Pneumococci transformed Type R Pneumococci into Type II forms. An extract from Type III Pneumococci transformed Type R into Type III forms. The transformed pneumococci produced the particular capsular specific polysaccharide characteristic of the type. It appears, therefore, as though some complex chemical substance had induced a change in the hereditary properties and definitely transformed one organism into a different but a closely related organism. This, if it is confirmed, is the first successful experiment of inducing a specifically directed genetic change in an organism by a chemical compound. All changes induced by radiation have been random or chance changes. Here a chemical compound from Type I organism *directs* a change in type to Type I organisms. The chemical compound from Type II *directs* the change to Type II, etc. Alloway, and Dawson and Sia vouch for the sterility of the extracts and for the absence of the derived type in the original culture of the Type R organisms. Their proof is accepted by Landsteiner. Since these experiments are of such importance in their genetic implications, they should be repeated, using cultures from single-cell isolates of the Type R organism. If such experiments confirm the reports, we would have the first demonstrated proof of a directed genetic change.

CHAPTER XXI

NITROGEN BASES

A discussion of the nitrogen bases need not necessarily be considered as a part of protein chemistry. It seems probable, however, either that the nitrogen bases represent metabolic products formed by the breaking down of amino acids in the plant or animal organism, or that amino acids enter into the synthesis of these compounds. The nitrogen bases, therefore, may be looked upon as either amino acid decomposition products or products which have been derived from amino acids through some vital process.

It will be impossible in the space at our disposal to consider all the nitrogen-containing compounds that have been isolated from plant or animal material. Only a few of the more important will be mentioned, and only a few of the reactions involved will be considered.

THE DECOMPOSITION PRODUCTS OF AMINO ACIDS.—Proteins are hydrolyzed by fungi and bacteria to their constituent amino acids which are then acted upon by the microorganisms to yield either bases or acids. Deamination occurs both with anaerobes and aerobes, the characteristic *aerobic* reactions being either the production of a saturated acid possessing one less carbon atom than the original amino acid or the production of a hydroxy acid having the same number of carbon atoms as the original amino acid.

$$R{-}CH(NH_2)COOH + O_2 = R{-}COOH + CO_2 + NH_3$$
$$R{-}CH(NH_2)COOH + H_2O = R{-}CH(OH)COOH + NH_3$$

In some instances the above reaction is modified, producing instead an alcohol with the elimination of carbon dioxide and ammonia. This reaction occurs[1] when amino acids are present in a medium where yeast is actively fermenting sugars, and has been used to prepare such rare alcohols as β-hydroxyphenylethyl alcohol (tyrosol), etc.,

$$\underset{\text{Tyrosine}}{OH(p)C_6H_4{-}CH_2{-}CH(NH_2)COOH} + H_2O = \underset{\text{Tyrosol}}{OH(p)C_6H_4{-}CH_2{-}CH_2OH} + NH_3 + CO_2$$

[1] Ehrlich, F., Über die Vergärung des Tyrosins zu *p*-Oxyphenyl-äthylalkohol (Tyrosol), *Ber.*, 44: 139–146 (1911); Ehrlich, F., and Pistschimuka, P., Überführung von Aminen in Alkohole durch Hefe- und Schimmelpilze, *Ber.*, 45: 1006–1012 (1912).

The characteristic *anaerobic* changes may be either (*a*) deamination and reduction or (*b*) decarboxylation. Deamination and reduction form a saturated acid containing the same number of carbon atoms, or deamination may take place without reduction and an internal rearrangement will cause the formation of an unsaturated acid.[2]

$$R—CH(NH_2)COOH + H_2 = R—CH_2COOH + NH_3$$
$$R—CH_2—CH(NH_2)COOH \rightarrow R—CH = CH—COOH + NH_3$$

Decarboxylation results in the formation of amines and is a typical reaction of the putrefying bacteria,

$$R—CH(NH_2)COOH \rightarrow R—CH_2—NH_2 + CO_2$$

or formic acid may be liberated, in which case reduction takes place with the formation of an amine having one less carbon atom than the original amino acid,

$$R—CH(NH_2)COOH + H_2 \rightarrow R—CH_2—NH_2 + H—COOH$$

Some workers believe that decarboxylation always involves both the above reactions, inasmuch as carbon dioxide and formic acid are usually formed simultaneously. The precise conditions for amine and acid formation under anaerobic conditions have not been accurately worked out. It seems probable that both these reactions proceed simultaneously, the preponderance of one or the other being determined by the particular type of organism which is involved. In only a few instances have the products of the reaction been investigated for some specific organism.

The fundamental differences between aerobic and anaerobic putrefaction may be stated as *the elimination of nitrogen and the formation of acids by aerobic organisms and the elimination of carbon dioxide with the formation of bases by anaerobic organisms.* It is recognized that there are exceptions to this rule. By and large, however, the rule will hold.

Certain of the amines produced from amino acids by bacteria are of importance. Arginine yields urea and ornithine; which in turn loses carbon dioxide to form *putrescine,* ($NH_2—CH_2—CH_2—CH_2—CH_2—NH_2$). Lysine loses carbon dioxide to form *cadaverine,* the next higher homolog of putrescine. These bases have similar properties. They were among the first compounds isolated from the putrefactive decomposition products of proteins and were originally classified as "ptomaines." The names are suggestive of undesirable compounds. The bases themselves are physiologically practically inert.

[2] Raistrick, H., On a New Type of Chemical Change Produced by Bacteria. The Conversion of Histidine into Urocanic Acid by Bacteria of the Coli-Typhosus Group, *Biochem. J.,* 11: 71–77 (1917).

Tyrosine on putrefactive decomposition loses carbon dioxide to form the base, *tyramine*, β(*p*-hydroxyphenyl)ethylamine, an active principle of ergot. Histidine similarly forms *histamine* (β-imidazoleethylamine); tryptophane forms *tryptamine* (β-indoleethylamine); and arginine forms *agmatine* (δ-guanidobutylamine). Tyramine, histamine, and tryptamine all have a powerful physiological action. They are violent poisons. Tyramine is more or less similar to epinephrine, in that it raises the blood pressure, whereas histamine reduces arterial pressure to the shock level (30–40 cm. Hg) even when used in dosages as low as 1 mg. per kilo body weight. Histamine has been found by Abel in the secretion of the skin of the African toad. Toad skin, according to Pliny, was a medicine. Abel showed that it contained a powerful drug. This is an additional instance where one of the old folk remedies was found to be not so foolish as it appeared to be.

Tyramine likewise occurs in the salivary gland [3] of the cephalopod *Octopus macropus*, and in the pods of the broom,[4] *Sarothamnus scoparius*. In the latter case it occurs in association with hydroxytyramine which had not been previously found to occur in nature.

Koessler and Hanke [5] made extensive investigations of proteinogenous amines. The earlier laboratory method of preparation involved decarboxylation by *Bacterium coli*. However, heating an amino acid with diphenylamine [6, 7] may serve as a general method for the preparation of the corresponding proteinogenous amine. Using this method, Abderhalden obtained 95 per cent of the theoretical amount of pure tyramine from tyrosine.

The odor of putrefying protein is due to indole and skatole (β-methylindole) derived from tryptophane, and is not due to the bases noted above.

We have already noted that certain of these bases were classified originally as "ptomaines" and were supposed to be responsible for ptomaine poisoning. Most of the bases, however, are physiologically

[3] Henze, M., Über den Tyramin- und Tyrosingehalt der Speicheldrüse der Cephalopoden; zugleich Methodisches zur Mikrobestimmung der beiden Substanzen, *Z. physiol. Chem.*, 182: 227–240 (1929).

[4] Schmalfuss, H., and Heider, A., Tyramin und Oxytyramin, blutdrucksteigernde Schwarzvorstufen des Besenginsters Sarothamnus scoparius Wimm, *Biochem. Z.*, 236: 226–230 (1931).

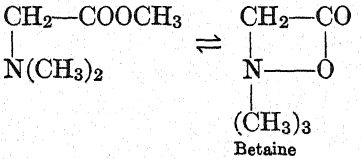
[5] Koessler, K. K., and Hanke, M. T., Studies on Proteinogenous Amines, I–V, *J. Am. Chem. Soc.*, 40: 1716–1726 (1918); *J. Biol. Chem.*, 39: 497–592 (1919); Koessler, K. K., The Relations of Proteinogenous Amines to Medicine, *Proc. Inst. Med. Chicago*, 3: 46–62 (1920).

[6] Johnson, T. B., and Daschavsky, P. G., Researches on Amines. IX. The Formation of Tyramine by Decarboxylation of Tyrosine Produced from Silk, *J. Biol. Chem.*, 62: 725–735 (1925).

[7] Abderhalden, E., and Gebelein, F., Über Decarboxylierung von Aminosäuren unter Bildung der entsprechenden Amine und über die Darstellung der Enolform von 2,5-Dioxo-piperazinen, *Z. physiol. Chem.*, 152: 125–131 (1926).

inert, and it is very doubtful if ptomaine poisoning is ever caused by any of these decomposition products of proteins. The common cause of ptomaine poisoning is the presence of the bacterial poison, *botulinus toxin,* secreted by *B. botulinus. Odor is no criterion of the presence or absence of botulinus toxin.* Completely rotted meat *may* be safe to eat, whereas apparently wholesome food may contain botulinus toxin, if opportunity has been afforded for the organism to develop. Botulinus toxin is destroyed by heat, which probably accounts for the relatively few cases of ptomaine poisoning which actually occur.

THE BETAINES.—The betaines are a group of bases found in plants, which can be defined as *completely methylated amino acids.* The α-, β-, and γ-amino acids form betaines. Those from α-amino acids are known as the α-betaines, those from β-amino acids as β-betaines, etc. The simple betaine is that of glycine, which is formed by an intramolecular rearrangement of the methyl ester of dimethylamino acetic acid,



Betaine

forming an internal salt, the nitrogen atom changing from the trivalent to the pentavalent condition. This betaine is physiologically inert. It occurs in the sugar beet and passes into the molasses in the process of sugar manufacture. Young sugar beets contain up to 2.5 per cent; old ones contain about 1 per cent of betaine. It likewise occurs in the leaves of many families of plants in quantities ranging from a fraction of 1 per cent to 3.78 per cent in *Atriplex canescens.*

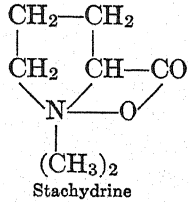
Barger [8] suggests that in plants betaines are end products of nitrogen metabolism and that they do not function in the vital processes. They are apparently most abundant in young or rapidly growing tissues.

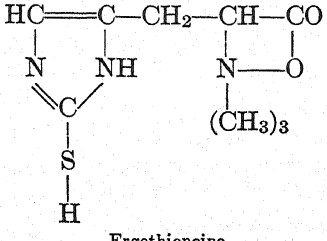
Stachydrine, the betaine of proline, occurs in the leaves of the orange tree to about 0.19 per cent of the dry weight. It is present [9] in the aqueous extract of alfalfa hay to the extent of about 0.5 per cent of the total nitrogen. Its chemical properties are such that in a Van Slyke analysis for nitrogen distribution it would be calculated in the *histidine* nitrogen fraction. Its physical properties are such, however, that using Kossel's method it would appear in the *lysine* fraction. These considerations emphasize the importance of recognizing the limitations of the various methods of protein analysis. *The methods of*

[8] Barger, G., The Simpler Natural Bases, Longmans, Green and Company, London (1914).

[9] Steenbock, H., Isolation and Identification of Stachydrin from Alfalfa Hay, *J. Biol. Chem.,* 35: 1–13 (1918).

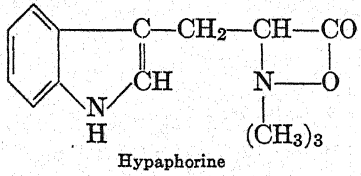
protein analysis are valid only when the material being analyzed is a pure protein. In the event that non-protein nitrogenous compounds are present, neither the Van Slyke method nor any other method based upon a nitrogen determination will necessarily yield accurate values for any particular amino acid.

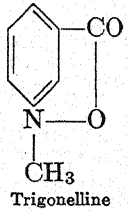

Stachydrine


Ergothioneine (Thiasine)

Benedict, *et al.*,[10] isolated a sulfur-containing nitrogenous base from blood and later showed this to be identical with the base *ergothioneine,* isolated by Tanret [11] from ergot and shown by Barger and Ewins [12] to be the betaine of thiolhistidine. Its function in the blood stream still remains to be determined. *Trimethyl histidine,* the betaine of histidine, is found in certain edible mushrooms. Its chemical properties are such that it would appear in the lysine fraction of the Kossel separation.

Hypaphorine, the betaine of tryptophane, has been found in the seeds of *Erythrina hypaphorus,* a shade tree grown on the coffee plantations of Brazil. It has a very slight physiological action.


Hypaphorine


Trigonelline

Trigonelline, the betaine of nicotinic acid, is widely distributed in plants. It occurs in the garden pea. The older literature states that trigonelline is physiologically inert. However, in view of the fact that vitamin properties (the "P-P factor," *vide infra*) have been ascribed

[10] Benedict, S. R., Newton, E. B., and Behre, J. A., A New Sulfur-Containing Compound (Thiasine) in the Blood, *J. Biol. Chem.*, 67: 267–277 (1926); Newton, E. B., Benedict, S. R., and Dakin, H. D., The Chemical Constitution of Thiasine, *Science*, 64: 602 (1926).

[11] Tanret, Ch., Sur une base nouvelle retirée du seigle ergoté, l'ergothionéine, *J. pharm. chim.*, (VI) 30: 145–153 (1909).

[12] Barger, G., and Ewins, A. J., The Constitution of Ergothioneine: a Betaine Related to Histidine, *J. Chem. Soc.*, 99: 2336–2341 (1911).

to nicotinic acid amide, it seems probable that trigonelline might be partially convertible in the animal organism into nicotinic acid, and thus acquire physiological activity. Williams [13] in his early studies on "vitamin B" investigated the physiological behavior of a number of pyridine derivatives including nicotinic acid and trigonelline. He notes that one crystal modification of trigonelline appeared to show curative effects of polyneuritic pigeons, but had no protective properties against polyneuritis. The "P-P factor" syndrome was unknown at that time, as was also the fact that the "vitamin B" then under investigation was in reality a complex containing several components.

It seems very possible that a betaine corresponding to each of the known amino acids may well occur in nature. However, aside from their interest as naturally occurring nitrogen-containing compounds, they appear to be of little biological importance.

CHOLINE AND ALLIED SUBSTANCES.—Certain bases are formed by the bacterial decomposition of substances other than proteins. Among these are *choline* and its natural allies, *neurine* and *possibly muscarine*. These are all strong bases. β-Amino ethyl alcohol is probably the precursor of choline, which is trimethyl-β-hydroxyethylammonium hydroxide, $(CH_3)_3—N(OH)—CH_2—CH_2OH$.

β-amino ethyl alcohol ($NH_2—CH_2—CH_2OH$) occurs as a constituent of lecithin from various sources. While the mechanism by which this base is formed is still uncertain, there is a possibility that it may be derived by the loss of carbon dioxide from serine.

$$OH—CH_2—CH(NH_2)COOH \rightarrow OH—CH_2—CH_2—NH_2 + CO_2$$

Acetylcholine has recently been demonstrated to be involved in the transmission of the nerve impulse to the ganglion cells, and also to transmit the motor nerve impulses to the fibers of our voluntary muscles. The pioneer work in the demonstration of the importance of acetylcholine in nerve reactions is due to Loewi, *et al.*,[14] who demonstrated the presence of a "Vagusstoff" as being liberated into the salt solution following the stimulation of the vagus nerve of the isolated frog heart. In a series of careful studies he identified his "Vagusstoff" with acetylcholine. Dale and Gaddum,[15] in 1930, extended Loewi's observation to the mammal, using dogs. They concluded that

[13] Williams, R. R., The Chemical Nature of the "Vitamines." III. The Structure of the Curative Modifications of the Hydroxypyridines, *J. Biol. Chem.*, 29: 495–520 (1917).

[14] Loewi, O., *et al*, Über humorale Übertragbarkeit der Herznervenwirkung, *Arch. ges. Physiol.*, 189: 239–242 (1921); 193: 201–213 (1922); 203: 408–412 (1924); 204: 361–367, 629–640, (1924); 206: 123–134, 135–140 (1924); 208: 694–704 (1925); 210: 550–556 (1926); 214: 678–688, 689–696 (1926); 217: 610–617 (1927); 225: 721–727 (1930).

[15] Dale, H. H., and Gaddum, J. H., Reactions of Denervated Voluntary Muscle, and Their Bearing on the Mode of Action of Parasympathetic and Related Nerves, *J. Physiol.*, 70: 109–144 (1930).

the nerve impulses were transmitted through the medium of acetylcholine. This mechanism of nerve impulse transport has been absolutely confirmed by subsequent studies.[16] Dale[17] comments, "As I speak to you, I have every reason to suppose that the muscle fibers of my tongue and my jaws are being activated by innumerable little charges of acetylcholine, fired at them, as it were, from the endings of the nerve fibers." He calculates that the transmission of a single nerve impulse to a single ganglion cell involves the liberation of approximately 3,000,000 molecules of acetylcholine.

Lecithin (a phospholipid) is a *choline glycerol phosphoric acid ester,* and inasmuch as it occurs in all cells and especially in nervous tissues, it must be of marked physiological importance. Putrefaction of lecithin yields choline as one of the products. Choline is a depressant, causing a fall in blood pressure, and is antagonized by the alkaloid, atropine. Choline may occasionally occur in the free state in plants. Vinson[18] isolated a considerable quantity of choline from a fraction of the dilute sodium hydroxide extract of corn pollen. Whether or not the choline was present in the free state in the corn pollen or represented a decomposition product of the lecithin was not determined.

When putrefying bacteria act upon choline, the alcoholic side chain suffers dehydration, forming *neurine,* vinyltrimethylammonium hydroxide, $(CH_3)_3—N(OH)—CH=CH_2$. Neurine is exceedingly poisonous and may occur among the putrefaction decomposition products of meat and fish. Further decomposition results in the formation of trimethylamine. Trimethylamine occurs[19] in the spores of *Tilletia levis,* the "stinking smut" of wheat.

According to the older literature, *muscarine* is the oxidation product of choline, where the alcohol group has been oxidized to an aldehyde. A recent investigation[20] of the chemistry of muscarine points to the probable formula

$$(CH_3)_3—N(OH)—CH(CHO)—CH(OH)CH_2—CH_3$$

which would be a β-hydroxyvalerianic aldehyde substituted in the α-position with the trimethylammonium hydroxide radical. It would thus be a higher homolog of the oxidation product of choline. Muscarine is the poison of the "fly mushroom" or "fly agaric," *Amanita*

[16] For review see Gaddum, J. H., Choline and Allied Substances, *Ann. Rev. Biochem.,* 4: 311–330 (1935).

[17] Dale, H., Chemical Ideas in Medicine and Biology, *Science,* 80: 343–349 (1934); *cf.* correction, *ibid.,* 80: 450 (1934).

[18] Vinson, C. G., Some Nitrogenous Constituents of Corn Pollen, *J. Agr. Research,* 35: 261–278 (1927).

[19] Hanna, W. F., Vickery, H. B., and Pucher, G. W., The Isolation of Trimethylamine from Spores of Tilletia Levis, the Stinking Smut of Wheat, *J. Biol. Chem.,* 97: 351–358 (1932).

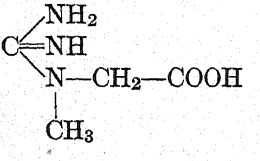
[20] Kögl, F., Duisberg, H., and Erxleben, H., Untersuchungen über Pilzgifte. I. Über das Muscarin. I, *Ann.,* 489: 156–192 (1931).

muscaria. This mushroom was Caesar's favorite poison because it resembles so closely *Amanita caesarea* which is edible. He accordingly had *Amanita caesarea* served to himself, and *Amanita muscaria* served on certain occasions to his guests. Muscarine poisoning is antagonized by atropine.[21]

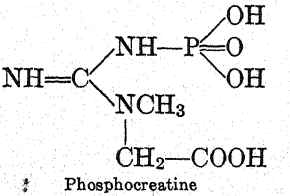
MISCELLANEOUS NITROGEN BASES.—Only a few of the nitrogen bases occurring in plants or animals can be considered. For a more adequate discussion the reader is referred to the book by Barger, already noted.

Guanidine occurs occasionally in plants. It is found in the sugar beet and passes into the molasses in the process of sugar manufacture. It is probably originally derived from arginine.

Creatine, methylguanidineacetic acid, is a constituent of all vertebrate muscle. It occurs in the urine during starvation and is an indication that muscle tissue is being broken down. Fiske and Subbarow [22] isolated a creatine phosphoric acid complex from muscular tissue and point out that this compound is present in considerable proportion in resting muscle but is rapidly decomposed in fatigued or dead muscle. They suggested [23] a physiological role for the creatine and phosphoric acid in the muscle, *i.e.*, that they act as a buffer to bind the lactic acid formed from sugars by muscular activity.



Creatine



Phosphocreatine

At approximately the same time that Fiske and Subbarow isolated their phosphocreatine, the Eggletons [24] isolated the same compound

[21] Warning should be inserted at this point, that while the poison of *Amanita muscaria* is antagonized by atropine, this is not the case for the "deadly amanita," *Amanita phalloides*. *Amanita phalloides contains the amanita toxin for which there is no antidote.* The toxin is a complex, possibly protein-like substance acting as a hemolysin, dissolving the red blood cells. It is one of the most poisonous of all plant products. One-half of an *Amanita phalloides* is sufficient for a fatal dose. As a rule, the symptoms of *Amanita* poisoning do not appear for some time after the mushroom has been eaten. For those who are interested in the properties of poisonous mushrooms, see Ford, W. W., and Clark, E. D., A Consideration of the Properties of Poisonous Fungi, *Mycologia*, 6: 167–191 (1914) (60 references to literature).

[22] Fiske, C. H., and Subbarow, Y., The Nature of the "Inorganic Phosphate" in Voluntary Muscle, *Science*, 65: 401–403 (1927).

[23] Fiske, C. H., and Subbarow, Y., The Isolation and Function of Phosphocreatine, *Science*, 67: 169–170 (1928).

[24] Eggleton, P., and Eggleton, G. P., The Inorganic Phosphate and a Labile Form of Organic Phosphate in the Gastrocnemius of the Frog, *Biochem. J.*, 21: 190–195 (1927).

and named it *phosphagen*. The reactions of this compound and a knowledge of the role which it plays in vital processes have revolutionized our concepts of muscle physiology.[25] The role which phosphocreatin plays in the phenomenon of muscle contraction involves a sequence of four stages:

1. On muscle stimulation phosphagen breaks down to creatine and phosphoric acid liberating 120 calories per gram phosphoric acid. *This is the primary reaction which provides energy for muscle contraction.*

2. As a result of the breakdown of phosphagen the acid-combining power of the tissue is increased, more at greater, less at lower, hydrogen-ion concentrations.

3. During and after muscle contraction, lactic acid is set free from the carbohydrate present in the muscle. In this process there is a liberation of 200 calories per gram lactic acid formed.

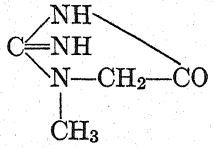
4. The phosphagen is resynthesized during muscle recovery. This is an *endothermic* process which is masked by the *exothermic* lactic acid formation. *The energy of lactic acid formation is used for the resynthesis of phosphagen.* In glycogen-free muscle or in one poisoned by iodoacetic acid this lactic acid formation does not take place and phosphagen is not resynthesized. In such muscles the initial contraction takes place normally but there is no recovery. When a muscle has been poisoned by iodoacetic acid, all the energy of contraction comes from the breakdown of phosphagen.

The above observations have overthrown the original lactic acid theory of muscle contraction and leave for lactic acid only the role of energy formation for phosphagen resynthesis. The appearance of lactic acid follows muscle contraction. It will be observed that the breakdown of phosphagen into phosphoric acid and creatine is a hydrolysis, but it should be pointed out that *it is not an ordinary hydrolysis.* Normally a hydrolysis involves practically no calorific changes. In this instance very appreciable quantities of heat are evolved. Perhaps this is because we have in phosphagen a direct bond between phosphorus and nitrogen. Perhaps the heat evolved is in part the heats of solution and of ionization of the phosphoric acid which is liberated. An attempt to ascertain just exactly what factors are responsible for the heat formed on the breakdown of phosphagen becomes even more complicated, if the Werner structure for creatine is considered.

While phosphocreatine appears to be the source of energy for the muscle contraction in the mammals, the corresponding arginine phosphoric acid ester is the active agent in crustacean muscle.

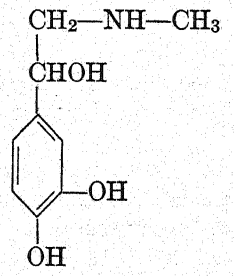
[25] Hill, A. V., The Revolution in Muscle Physiology, *Physiol. Rev.*, 12: 56–67 (1932); *cf.* also Milroy, T. H., The Present Status of the Chemistry of Skeletal Muscle Contraction, *Physiol. Rev.*, 11: 515–548 (1931); and Needham, D. M., The Biochemistry of Muscle, 166 pp., Methuen and Company, London (1932).

Creatinine, the anhydride of creatine, occurs normally in all urine. The ratio of creatine to creatinine has been extensively studied as indicating relationships to physiological conditions of the individual. A discussion of such relationships, however, properly belongs in textbooks of physiological chemistry.

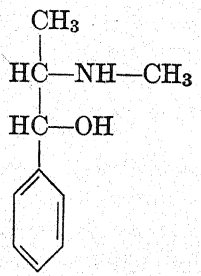


Creatinine

Another base of the utmost importance has three names which are rather common in literature, *epinephrine* (Abel), *suprarenine* (Von Fürth), and *adrenaline* (Takamine). Since this base was first isolated by Abel, it should be known as epinephrine. Epinephrine is a derivative of pyrocatechol. It will be noted that the formula shows a marked resemblance to tyrosine. Undoubtedly epinephrine is one of the most important of all the naturally occurring bases. It is one of the active principles of the adrenal glands and regulates the blood pressure in mammals. In health, about 0.1 per cent of the gland is epinephrine. The natural product is *l*-rotatory. The racemic mixture, containing the *d*- and *l*-modifications in equivalent amounts, has a much lower physiological activity than the natural product, the *d*-form having only approximately 5 per cent of the physiological activity of the *l*-form, again a striking example of biological specificity. An injection of as little as 0.0003 mg. per kilo of body weight is sufficient to produce a marked effect on blood pressure. The lethal dose (for man) is about 0.06 gram when injected intravenously. It contracts the blood vessels so that no blood can flow. Because of this marked contractile effect, epinephrine has come to be a valuable adjunct in surgery to control bleeding during a surgical operation. Together with other physiologically active constituents, epinephrine occurs in the secretions of two African toads, *Bufo regularis* and *B. arenarum.*[26]



Epinephrine



Ephedrine

[26] Jensen, H., Chemical Studies on Toad Poisons. VII. Bufo arenarum, Bufo regularis and Zenopus laevis, *J. Am. Chem. Soc.,* 57: 1765–1768 (1935).

Ephedrine, another base very similar in structure to epinephrine and having the formula [27] noted, occurs in the plant *Ephedra equisetina,* and in smaller amounts in the leaves of the yew, *Taxus baccata.* This base has many of the desirable properties of epinephrine. It raises the blood pressure and has the advantage that it can be taken orally.

Two synthetic products combining portions of the structure of both epinephrine and ephedrine have proved of value in medicine. These are "corbasil" or "cobefrin" and "benzedrine." Corbasil is apparently β-hydroxy-β′-(3.4-dihydroxyphenyl)isopropylamine, and benzedrine is β-phenylisopropylamine. Benzedrine has a stimulating effect on the higher centers of the central nervous system and has been used with good results in cases of nervous fatigue, chronic exhaustion, psychoneuroses, and manic-depressive psychoses.

3-4-Dihydroxyphenylalanine or *dopa* is an amino acid similar in some respects to epinephrine in structure. It occurs in the Georgia velvet bean (*Stizolobium deeringianum*) and in the seeds of *Vicia faba.* It reacts more or less like epinephrine but does not have the highly marked physiological properties, although it is distinctly toxic. It has not as yet been isolated from a protein. It is apparently the *chromogen* involved in certain brown and black animal pigmentations,[28] such as in butterfly wings, *e.g., Vanessa antiopa.* In *Tenebrio molitor,* the chromogen [29] appears to be 3.4-dihydroxyphenylacetic acid, which presumably arises from dopa.

Carnosine, the dipeptide, β-alanylhistidine, has already been referred to. It is one of the principal products in "beef extracts," and may be looked upon either as a dipeptide or as a base which is a constituent of muscle.

Anserine, a dipeptide of β-alanyl-N-methylhistidine, where the methyl group is attached to the nitrogen of the imidazole ring, occurs among the extractives of the flesh of the dog, cat, rabbit, white rat, goose, chicken, turkey, pigeon, and crocodile. It has not been isolated from the muscle protein. The fact that it contains an N-methyl group may account for a part or all of the N-methyl compounds which have been reported to occur in proteins.

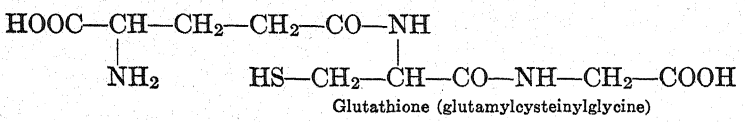
Glutathione, a tripeptide, glutamylcysteinylglycine, was isolated

[27] Peterson, Joel B., Standardization of Ephedrine and Its Salts, *Ind. Eng. Chem.,* 20: 388–391 (1928).

[28] Hasebroek, K., Untersuchungen zum Problem des neuzeitlichen Melanismus der Schmetterlinge, I–X, *Fermentforschung,* 5: 1–40, 297–333 (1921–22); 7: 1–13, 139–142, 143–152, 183–194 (1923–24); 8: 197–198, 199–226, 553–567, 568–573 (1925–26).

[29] Schmalfuss, H., Heider, A., and Winkelmann, K., 3,4-Dioxyphenylessigsäure, Farbvorstufe der Flügeldecken des Mehlkäfers, Tenebrio molitor L., *Biochem. Z.,* 257: 188–193 (1933).

by Hopkins [30] from yeast, muscle, and mammalian liver. Such a tripeptide has twelve possible structural formulas. Kendall [31] has shown that glutathione has the formula



Glutathione (glutamylcysteinylglycine)

This appears to be the substance which is present in all cells that give the nitroprusside test. It is generally believed that glutathione acts as a hydrogen receptor and takes an active part in cell oxidations and reductions: [32]

$$2\ R{-}SH \rightleftharpoons R{-}S{-}S{-}R + H_2$$

Kendall and Nord [33] suggest that an unstable highly reactive oxygen addition product is formed between glutathione and oxygen, in which product the sulfur atom has a higher state of oxidation. This compound, together with the more stable —SH and —S—S—forms, make up a reversible oxidation-reduction system.

More recently attention has been called to the role which glutathione may play in intermediary protein metabolism.[34] The only three amino acids which seem to be available for the process of detoxification in the human are the same as those which form glutathione. It is suggested that the mechanism may be that the substance being detoxified first combines with glutathione, and then this compound breaks down, leaving a part of the original glutathione molecule associated with the toxic compound.

[30] Hopkins, F. G., On an Autoxidisable Constituent of the Cell, *Biochem. J.*, 15: 286–305 (1921); On Glutathione: A Reinvestigation, *J. Biol. Chem.*, 84: 269–320 (1929).

[31] Kendall, E. C., McKenzie, B. F., and Mason, H. L., A Study of Glutathione. I. Its Preparation in Crystalline Form and Its Identification. *J. Biol. Chem.*, 84: 657–674 (1929). *Cf.* also "The Configuration of Glutathione," *Proc. Staff Meetings Mayo Clinic*, 4: 359–360 (Dec. 11, 1929).

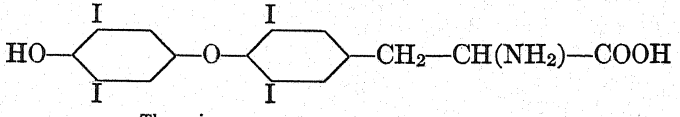
[32] Dixon, M., and Quastel, J. H., A New Type of Reduction-Oxidation System. Pt. I. Cysteine and Glutathione, *J. Chem. Soc.*, 123: 2943–2953 (1923); *cf.* also Quastel, J. H., Stewart, C. P., and Tunnicliffe, H. E., On Glutathione. IV. Constitution, *Biochem. J.*, 17: 586–592 (1923); Tunnicliffe, H. E., Glutathione. The Occurrence and Quantitative Estimation of Glutathione in Tissues, *Biochem. J.*, 19: 194–198 (1925); Stewart, C. P., and Tunnicliffe, H. E., Glutathione. Synthesis, *Biochem. J.*, 19: 207–217 (1925).

[33] Kendall, E. C., and Nord, F. F., Reversible Oxidation-Reduction Systems of Cysteine-Cystine and Reduced and Oxidized Glutathione, *J. Biol. Chem.*, 69: 295–337 (1926).

[34] Brand, E., and Harris, M. M., Some Aspects of Intermediary Protein Metabolism, *Science*, 77: 589–590 (1933).

Numerous reports have demonstrated that the —SH group is effective in stimulating growth of epithelial tissue and in promoting granulation of wounds. It may be that a very considerable part of the biological effects of glutathione reside in the stimulation afforded tissues by the —SH linkage. In the case of malignant growth, spontaneous mammary carcinomas are in a large measure controlled [35] by a diet deficient in cystine or methionine. Following such repression, the growth of the carcinoma is greatly stimulated by either cystine or glutathione.

Spermine, isolated by Rosenheim,[36] has been shown [37] to be α-δ-bis-(γ′-aminopropylamino)butane, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$. It is apparently a constant constituent of sperm, although it has been found to occur in other animal organs. A similar compound, *spermidine,*[38] α-(γ′-aminopropylamino)-δ-aminobutane, $NH_2(CH_2)_3NH(CH_2)_4NH_2$, occurs in association with spermine in the various tissues. The physiological function of these bases has not been determined.



Thyroxine

Thyroxine, the hormone of the thyroid gland, was isolated by Kendall [39] as a decomposition product of the protein of the thyroid gland. It has been synthesized [40] by known reactions. Thyroxine has a very marked physiological action, exceptionally small amounts increasing the metabolism of the body. As little as 1 mg. of thyroxine injected

[35] Voegtlin, C., Johnson, J. M., and Thompson, J. W., Glutathione and Malignant Growth, *U. S. Pub. Health Repts.,* 51: 1689–1697 (1936).

[36] Rosenheim, O., The Isolation of Spermine Phosphate from Semen and Testis, *Biochem. J.,* 18: 1253–1262 (1924).

[37] Dudley, H. W., Rosenheim, O., and Starling, W. W., The Chemical Constitution of Spermine, III. Structure and Synthesis, *Biochem. J.,* 20: 1082–1094 (1926).

[38] Dudley, H. W., Rosenheim, M. C., and Rosenheim, O., The Chemical Constitution of Spermine. I. The Isolation of Spermine from Animal Tissues, and the Preparation of Its Salts, *Biochem. J.,* 18: 1263–1272 (1924); Dudley, H. W., Rosenheim, O., and Starling, W. W., The Constitution and Synthesis of Spermidine, a Newly Discovered Base Isolated from Animal Tissues, *Biochem. J.,* 21: 97–103 (1927).

[39] Kendall, E. C., Isolation of the Iodine Compound Which Occurs in the Thyroid, *J. Biol. Chem.,* 39: 125–147 (1919); Influence of the Thyroid Gland on Oxidation in the Animal Organism, *Ind. Eng. Chem.,* 17: 525–534 (1925).

[40] Harington, C. R., Chemistry of Thyroxine. I. Isolation of Thyroxine from the Thyroid Gland, *Biochem. J.,* 20: 293–299 (1926); II. Constitution and Synthesis of Desiodo-Thyroxine, *Biochem. J.,* 20: 300–313 (1926); (and Barger, G.), III. Constitution and Synthesis of Thyroxine, *Biochem. J.,* 21: 169–183 (1927).

intravenously may increase the metabolic rate as much as 2.5 per cent or, as Kendall puts it, "The injection of 1 milligram of thyroxine will produce an increase in the carbon dioxide output of approximately 400,000 milligrams of carbon dioxide."

Following the injection of thyroxine, there is a pronounced delay in reaction, 6 to 8 hours being required before the basal metabolism rate is affected. Subsequently there is a rapid increase in metabolic rate, but the maximal response may not be reached for several days after the injection. In a number of instances the maximal response to a single injection was not reached until the eighth or tenth day after the injection. The physiological effect of most drugs is over within a relatively short period of time. Thus, the effect of epinephrine may last, at the most, only a few hours. Kendall notes that a single injection of 5 to 10 mg. of thyroxine may affect the basal metabolic rate for a period as long as 5 to 6 weeks.

When certain pathological conditions influence the function of the thyroid gland so that thyroxine is no longer synthesized, as in the case of myxedema and cretinism, the entire physiological process undergoes alteration which is particularly noted in the mental reactions, the individual either remaining essentially an idiot (cretinism) or losing his mental faculties (myxedema). In each instance the mental faculties can be more or less completely restored and physiological processes brought back to normal by means of thyroxine. Accordingly the isolation of this hormone in a form suitable for use in medicine ranks as one of the great contributions to modern medicine.

THE ALKALOIDS.—Among the vegetable products, numerous oily or crystalline bases have been found, to which the term "alkaloid" has been applied, and because of their physiological properties they have, for ages past, interested mankind.

When the orientals used opium or hashish, or the South American aborigines chewed the coca leaves for stimulation or the cinchona bark for fevers, they did not know that the reason they secured results lay in the alkaloidal content of the material. Only within the last few decades has the organic chemistry of certain of the alkaloids been elucidated. Owing to the complex structure of the alkaloidal molecule, a study of the chemistry of the alkaloids is one of the most difficult fields of organic chemistry, and even today the structural formulas of such important drugs as strychnine and quinine are still more or less uncertain.

We can only consider briefly a few of the more important alkaloids. For those who are interested in the further development of this subject reference may be made to a number of books.[41-47]

[41] Henry, T. A., The Plant Alkaloids, P. Blakiston's Son and Company, Philadelphia (1913).

It is difficult to formulate an exact definition of an alkaloid. The definition may be made so broad as to include all nitrogen-containing compounds or so narrow as to leave out compounds with definite alkaloidal properties. Ladenberg originally defined them as *"those naturally occurring vegetable substances of a basic character which contain at least one nitrogen atom forming a part of a heterocyclic ring."* If we except the purine and pyrimidine bases, we can limit the definition to "basic substances found in plants and which contain a cyclic nitrogenous nucleus." Even this is too narrow, for a few compounds do not contain a "cyclic nitrogenous nucleus," nevertheless they may have the marked physiological properties of alkaloids. The modern chemist may well take exception to the words, "naturally occurring." We have prepared synthetically medicinal substances which are *better* than the natural alkaloids, and these synthetic products are truly alkaloidal, both in chemical and physiological properties, if we accept chemical and physiological properties as criteria. Perhaps the recent definition of Thompson,[48] *"a relatively complex organic chemical entity containing nitrogen, whose free base is alkaline in reaction and capable of neutralizing acids to form salts, whose free base is much less soluble in water than its salts, whose free base is much more soluble in ether, chloroform, benzol, etc., than its salts, and as a substance whose salts in solution release the free base upon the addition of alkalies,"* is as satisfactory as any which can be devised.

Historically, the work on alkaloids dates back to 1803 when Derosne isolated a crystalline compound from opium, which he called "opium salt." He did not, however, notice its basic character. In 1805, Sertürner, a German apothecary, isolated the material again independently, purified it, recognized its basic properties, and called it "morpheum." At the same time he separated an acid which he called "meconic" acid, and expressed the view that the two were combined in opium. These observations remained unnoticed until 1817, when Sertürner published a second paper, in which he further pointed

[42] Pictet, A., The Vegetable Alkaloids, translated from the French by H. C. Biddle, John Wiley & Sons, New York (1904).

[43] Sidgwick, N. V., The Organic Chemistry of Nitrogen, New Edition, revised and rewritten by T. W. J. Taylor and Wilson Baker, Clarendon Press, Oxford (1937).

[44] Spiegel, L., Der Stickstoff, Fr. Vieweg und Sohn, Braunschweig (1903).

[45] Winterstein, E., and Trier, G., Die Alkaloide, Gebrüder Borntraeger, Berlin (1910).

[46] Schmidt, J., and Grafe, V., Alkaloide, Urban und Schwarzenberg. Berlin (1920).

[47] Blyth, A. W., Poisons: Their Effects and Detection, Third Edition. Charles Griffin and Company, Ltd., London (1895).

[48] Thompson, M. R., The New Active Principle of Ergot, *Science*, 82: 62–63 (1935).

out the basic character of morphine and described a number of its salts. Chemists then began to look for other similar compounds, and in 1818, Pelletier and Caventou found strychnine in *Nux vomica*, followed by brucine in 1819, and in 1820 they isolated quinine and cinchonine from cinchona bark. At least two or three new alkaloids have been isolated and described each year since 1820.

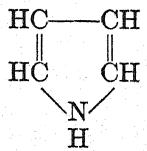
There was considerable speculation by the early chemists, as to the chemical constitution of these compounds, but the first definite clue was obtained about 1842–1846. Gerhardt, in 1842, distilled quinine, strychnine, and cinchonine with solid potassium hydroxide and obtained an oily base which he called "quinoleine"; later the name was changed to quinoline.

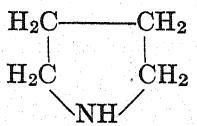
Previous to this, Runge (1834) had obtained a base from coal tar which he called "leucol," and Hoffman found that "quinoleine" and "leucol" were identical. Meanwhile, Andersen (1849–1851) separated pyridine from bone oil, and later this base was isolated from the alkaloids, nicotine, coniine, piperidine, etc., by distillation with zinc dust. Isoquinoline was isolated from coal tar in 1885, and was later obtained from certain of the alkaloids, hydrastine, papaverine, etc. The fact that coal-tar bases could also be obtained from alkaloids stimulated research, and by breaking down alkaloids on the one hand, and building up derivatives of the coal-tar bases on the other, points of contact were established and information regarding the alkaloidal molecule was obtained. Eventually, some of the alkaloids were synthesized, and the recent advance in this field has been in the discovery of the *potent groupings* and then using these new groupings in new compounds which are better than the naturally occurring alkaloids, in that *they have the same curative or medicinal properties and less of the undesirable properties.*

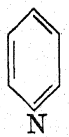
In using alkaloids in medicine, two properties of the alkaloid must be taken into consideration, *i.e.*, the minimal medicinal dose and the minimal lethal dose. The minimal medicinal dose is that dosage which will bring about the desired physiological effect. The minimal lethal dose is that dosage which may cause death. A number of alkaloids having very desirable physiological properties cannot be used or can be used only with great caution, because of the fact that their physiological dose lies very close to their toxic dosage. Sometimes only three or four times the physiological dose is sufficient to cause death. The aim of the synthetic organic chemist interested in the field of the alkaloids is to ascertain those groupings which give to an alkaloidal molecule medicinal properties and those groupings which give to the alkaloid its toxic properties, with the hope of being able to synthesize new organic compounds having the desirable properties and lacking the undesirable properties of the naturally occurring alkaloids. The synthesis of *novocaine*, *procaine*, *butyn*, etc., has been accomplished by

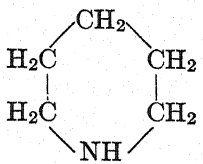
studying the molecular configuration of cocaine and attempting to synthesize local anesthetics having a similarity to certain parts of the cocaine molecule but lacking the toxic groupings.

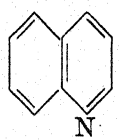
A classification of the alkaloids has been made on the basis of the heterocyclic nucleus, although more than one heterocyclic ring may be present in the molecule of a single alkaloid. The following ring structures are the characteristic structures found in naturally occurring alkaloids.

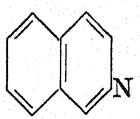

Pyrrole


Pyrrolidine


Pyridine


Piperidine


Quinoline


Isoquinoline

In most discussions of alkaloids, the *purine derivatives*, such as caffeine and theobromine, are considered as belonging to the alkaloid group. We have already noted these compounds as derivatives of the purine nucleus under nucleic acids and will not include them again at this point.

Before considering the organic chemistry of the alkaloids, it is perhaps justifiable to note certain of the families of plants in which alkaloids occur. Alkaloids are very unevenly distributed throughout the various groups of the plant kingdom. In some families of plants many alkaloids are found. Other families are noted by the practical absence of alkaloids. Very few of the monocotyledonous plants contain alkaloids, these compounds being confined almost exclusively to the dicotyledonous plants.

Among the angiosperms six families are noteworthy for their alkaloidal content:

1. The *Apocynaceae* —"dogbane" (tropic and subtropic);
2. The *Leguminosae* —legumes;
3. The *Papaveraceae* —poppies;
4. The *Ranunculaceae*—buttercup and crowfoot;
5. The *Rubiaceae* —madder;
6. The *Solanaceae* —potato, tomato, nightshade.

Other families, such as the mints, roses, orchids, etc., sometimes, though rarely, contain alkaloids. They may occur in cell sap (opium), leaves (coca), stems, fruits (piperine, black pepper), seeds (*Nux vomica*), bark (*quinine*), roots (berberine in barberry roots), etc. Rarely does one alkaloid occur alone; usually two or three or more occur together; opium contains at least 20, and new ones are still being isolated from opium.

The alkaloids are usually solid and crystalline, but a few, like nicotine and coniine, are liquids. They are mostly colorless, although a few are yellow. They rarely occur free in the plant tissue, but as salts of organic acids, *e.g.*, malic, citric, succinic, oxalic, tannic, quinic, meconic, or aconitic, etc. In several groups of plants special alkaloids occur with special acids. Thus, the aconite alkaloids occur combined with aconitic acid, the opium alkaloids with meconic acid, and the cinchona alkaloids with quinic acid. Alkaloids readily form crystallizable salts with inorganic acids and are extracted from plant tissues by dilute sulfuric acid or hydrochloric acid. Certain of the alkaloids are volatile and may be steam-distilled from alkaline media; the nonvolatile ones are set free by sodium hydroxide and extracted with ether, chloroform, etc., or may be adsorbed on Lloyd's reagent.

McNair,[49] in a series of papers directed toward a study of plant phylogeny, has surveyed the distribution and physical properties of the acids, alcohols, glycerides, essential oils, and alkaloids, as they occur in various plant families, with particular reference to the climatic distribution of the compounds. He finds that alkaloids occur in 57 of the 295 families of angiosperms and gymnosperms. Forty-four per cent of the families are mostly tropical; 14 per cent are wholly temperate. Two of the 5 families of the gymnosperms, 5 of the 45 families of monocotyledonous plants, and 44 of the 241 families of the dicotyledonous plants contain alkaloids. The same alkaloid is seldom found in different plant families. On the other hand, a particular alkaloid may be often found in a number of members of a single plant family. When different alkaloids occur in the same plant family, the alkaloid is usually confined to a single genus. In a study of the tropical alkaloids, McNair concludes that the higher the plant is in the scale of evolutionary development, the higher will be the molecular weight of the alkaloid which the family contains. He further points out that the melting point of the tropical alkaloids is, in general, higher than the melting point of the subtropical alkaloids, and these in turn have a higher melting point than those of the temperate alkaloids. A

[49] McNair, J. B., Some Properties of Alkaloids in Relation to Climate of Habitat, *Am. J. Botany*, 18: 416–423 (1931); The Evolutionary Status of Plant Families in Relation to Some Chemical Properties, *Am. J. Botany*, 21: 427–452 (1934); The Taxonomic and Climatic Distribution of Alkaloids, *Bull. Torrey Botan. Club*, 62: 219–226 (1935); Angiosperm Phylogeny on a Chemical Basis, *ibid.*, 62: 515–532 (1935).

similar observation holds for the other compounds (glycerides, acids, etc.) investigated, and he suggests that chemical studies of plant constituents may be an aid in tracing evolutionary developments.

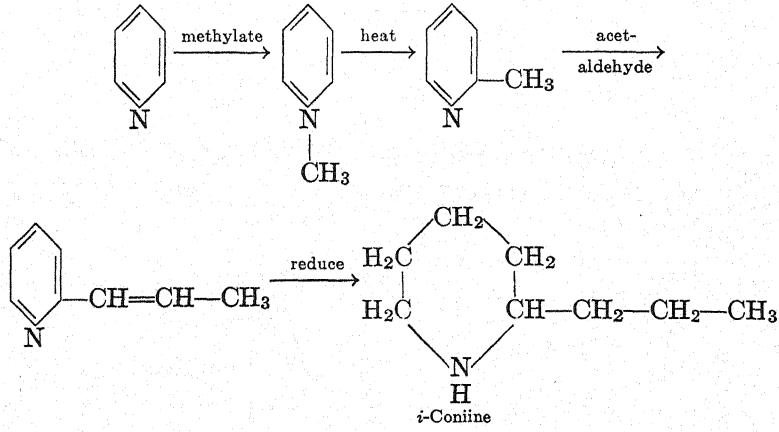
Various tests are used to detect the presence of alkaloids. Among the more common are: iodine in potassium iodide forming a yellow-brown precipitate (Wagner's reagent), platinic chloride forming a crystalline double salt which can be analyzed for platinum content, auric chloride forming a crystalline aurichloride (a similar crystalline double salt), and lead acetate yielding a precipitate.

Most alkaloids are bitter, but this is not a necessary property, for piperine, from black pepper, is tasteless. (The pungent taste of pepper is *not* due to its alkaloidal content but to an essential oil.)

Many of the alkaloids contain an asymmetric carbon atom and are accordingly optically active. Most of them are *l-rotatory*. *d*-Tartaric acid is commonly used to resolve racemic mixtures, following the same technic as already noted for resolving amino-acid mixtures.

The Pyridine Group.—This group includes nicotine (tobacco), coniine (hemlock), piperine (black pepper), etc.

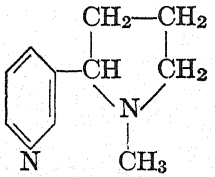
Coniine is α-*n*-propylpiperidine. This was the first alkaloid to be synthesized, Ladenberg, in 1886, accomplishing the synthesis.



Pyridine was methylated on the nitrogen, forming *n*-methylpyridine, which when heated caused a migration of the methyl group to the α position. The α-picoline so formed condensed with acetaldehyde to form α-allylpyridine, which on reduction yielded inactive coniine. The racemic mixture was separated by crystallization with *d*-tartaric acid. It would appear as if the synthesis could have been easily accomplished by adding a normal propyl group at the stage where the $-CH_3$ group was added to the pyridine. Ladenberg attempted to do this and found that, when the alkyl radical shifted from the nitrogen

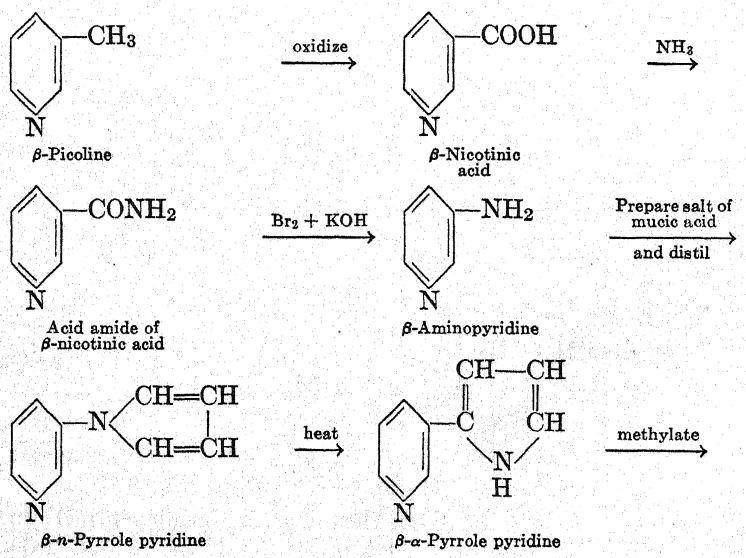
to the α-carbon, it became an isopropyl group, thus resulting in the synthesis of an isomer of coniine. From the historical standpoint coniine is an interesting alkaloid, inasmuch as Socrates was supposed to have died from the effect of coniine in his drink of the deadly hemlock.

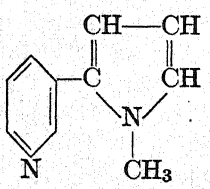
Nicotine, the alkaloid of tobacco, is β-(*n*-methylpyrrolidine)pyridine. This alkaloid was synthesized by Amé Pictet during the period



Nicotine

1895–1904, starting from β-picoline (β-methylpyridine) which is formed when glycerol, an organic nitrogen compound, and P_2O_5 are dry distilled. The mechanism of the reaction is unknown. The various steps in Pictet's synthesis are as follows:



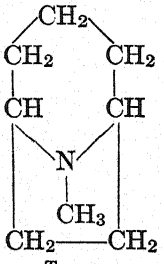


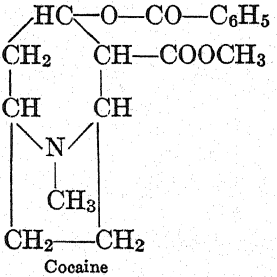
Nicotyrine

Nicotyrine treated with iodine forms the diiodo compound which reduces with zinc and potassium hydroxide to the dihydro compound. The dihydro compound with bromine gives the dibrom inactive nicotine, and the bromine can be removed from this by reducing with tin and hydrochloric acid, forming *inactive nicotine* which can be separated into its optical isomers. The trick in this synthesis was the reduction of the nicotyrine, reducing the pyrrole ring without reducing the pyridine ring. Solving the problem of reduction required nearly ten years of research.

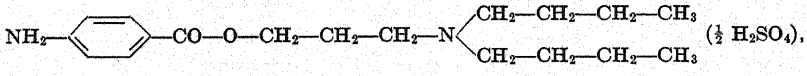
The Pyrrolidine Group.—The pyrrolidine group includes relatively few alkaloids. Only one of these is of any interest to us, *i.e.*, *stachydrine* which has already been mentioned as the betaine of proline.

The Tropane Group.—The tropane group contains a ring composed of a 6-membered piperidine ring and the 5-membered pyrrolidine ring, and can be regarded as derivatives of the heterocycle, *tropane*. These alkaloids are found in the *Solanaceae* and in cocoa, and are represented by *atropine, hyoscine, hyoscyamine, cocaine*, etc.

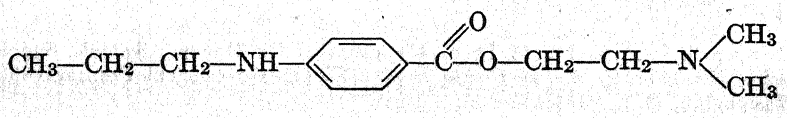

Tropane


Cocaine

Cocaine has been synthesized by Willstätter. Attempts to synthesize compounds having the desirable properties have yielded *novocaine* $(C_2H_5)_2N—CH_2—CH_2—O—CO—C_6H_4—NH_2$ (*p*), which is only one-seventh as toxic as cocaine. The local anesthetic properties of cocaine lie in the benzoic acid ester and nitrogen portion. Undesirable properties accompany the methyl ester group. Novocaine cannot be used as a surface anesthetic. *Butyn*,


($\frac{1}{2}$ H_2SO_4),

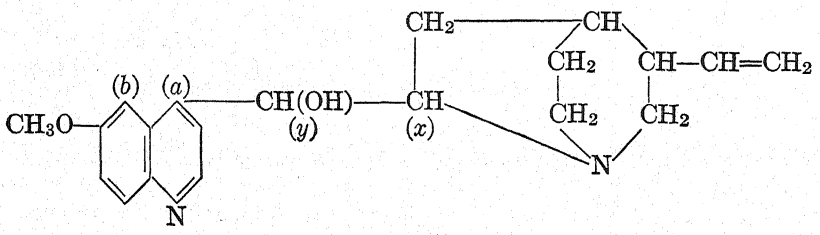
however, is fairly efficient in this regard. *Pantocain*, another synthetic local anesthetic, and one which has had considerable use in spinal anesthesia, apparently has the structure



For injection into the body it is desirable to have a water-soluble, neutral material, therefore, in the synthetic products the p-NH_2 group is added to the benzene nucleus. The hydrochloride of the synthetic compounds containing the p-amino group is essentially neutral.

The Quinoline Group.—This includes the alkaloids, *quinine, cinchonine, strychnine, brucine,* etc. None of these alkaloids have been synthesized, so their structures are not certainly known.

Quinine is probably



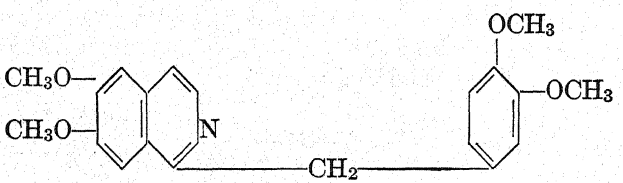
The OH at (y) may be at (x).

The union at (a) may be at (b).

If $—OCH_3$ is on the quinoline nucleus, the compound is *quinine*; if —OH is on the quinoline nucleus, the compound is *cuprine*, while if —H is on the quinoline nucleus, the compound is *cinchonine*. *Ethyl hydrocupreine* has OC_2H_5 on the quinoline nucleus and the $—CH=CH_2$ group is hydrogenated to $—CH_2—CH_3$. It has very desirable properties as a specific for *pneumococcus meningitis*, but the lethal dose is dangerously close to the curative dose, so that it is used only in an emergency.

The exact formulas for *strychnine* and *brucine* are more imperfectly known than that for quinine. Brucine is dimethoxy strychnine.

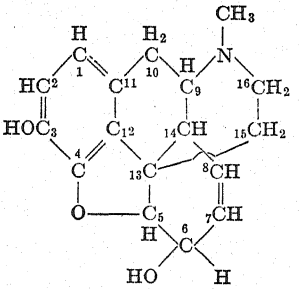
The Isoquinoline Group.—The isoquinoline alkaloids occur mainly in the opium series.[50]



Papaverine

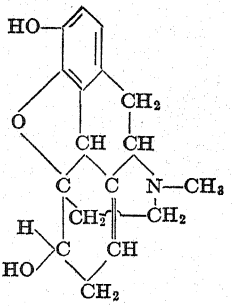
Papaverine is one of the simplest of these; *morphine* is probably one of the most complex.

[50] Small, L. F., and Lutz, R. E., Chemistry of the Opium Alkaloids, Supplement 103 to the *U. S. Pub. Health Repts.*, 375 pp., Government Printing Office, Washington, D. C. (1932).



(Robinson, 1925)

Two suggested alternative formulas for morphine ($C_{17}H_{19}NO_3$)



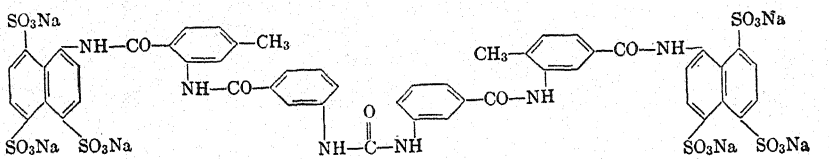
(Knorr-Hörlein, 1907)

Small notes that the Robinson formula appears to satisfy all the known reactions and decomposition products of morphine and that it may be accepted provisionally as indicating the structural relations of the more complex opium alkaloids. The problem of the opium alkaloids is complicated by the fact that there are a number of asymmetric carbon atoms in the molecule. Thus, in the morphine formula of Robinson there are five asymmetric carbon atoms, and Small notes that carbons -5, -6, and -9 are levorotatory and carbon-13 and carbon-14 are dextrorotatory. Therefore, the difficulties of attempting the synthesis of such a complicated structure are almost insuperable because the molecule contains four 6-membered rings and one 5-membered ring, and even if such a structure were synthesized, one would still face the problem of securing the proper space configurations on five asymmetric carbon atoms.

In discussing the phenomenon of molecular orientation and the influence of specific molecular configuration on orientation at interfaces, we have already referred to the change in physiological properties brought about by the conversion of the —OH group on the benzene ring of morphine to a carbonyl group and the simultaneous conversion of the benzene ring into a dihydrobenzene derivative. This synthetic compound, *dihydromorphinone* (dilaudid), is much more effective for the relief of pain, and the psychic and habit-forming effects of morphine are greatly reduced.

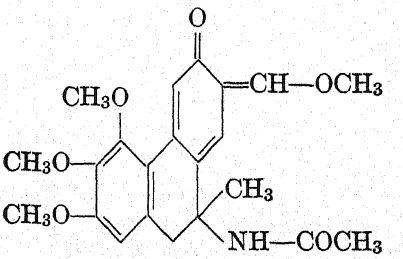
The announcement of a specific for the African sleeping sickness is the result of organic chemical research, not based upon the study of any particular alkaloid. The question accordingly arises as to whether or not mention of such a compound ought to be made in a section devoted to alkaloids. It seems to the author, however, that a synthetic product possessing more or less of an alkaloidal type of structure might well be considered, particularly in view of its immense importance in combating the ravages of the tsetse fly in certain regions of Africa. It is estimated that $500,000 was spent in experimental research by the Baeyer Company of Germany before "Baeyer 205" was perfected.

"Baeyer 205" appears to be identical in formula with the French "Fourneau 309," and to have the formula:



The therapeutic dose is only 1/160 of the lethal dose. It is, therefore, reasonably safe to administer. It is water-soluble, apparently cures the "sleeping sickness," and is stated to confer an immunity which lasts for months.

One unusual alkaloid deserves special mention. This is *colchicine*, an alkaloid obtained from *Colchicum autumnale*. This alkaloid is interesting from two standpoints. In the first place it does not contain a nitrogen in a heterocyclic ring but is in reality an acetylated amine of a substituted tetrahydrophenanthrene.



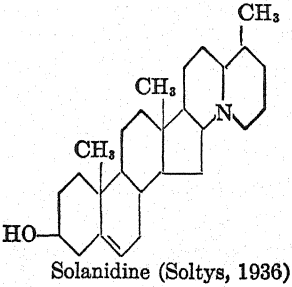
Colchicine ($C_{22}H_{25}O_6N$)
(Probable formula)

Its interest from the standpoint of biology lies in the fact that Blakeslee[51] found that when plants are treated with colchicine there is a doubling of the chromosome number so that tetraploids are produced from diploids. Tetraploid plants have in each organ approximately the same number of cells as diploid plants. The doubling of the chromosomes, however, results in the approximate doubling of the cell volume. Similarly octaploid plants having four times the chromosome number of diploid plants would have cell volumes approximately four times as great as those of the diploid type. The implications of polyploidy for the horticulturist are obvious, for the flowers and fruits of the polyploid varieties would be much larger than

[51] Blakeslee, A. F., Dedoublement du nombre de chromosomes chez les plantes par traitement chimique, *Compt. rend.*, 205: 476–479 (1937); Blakeslee, A. F., and Avery, A. G., Methods of Inducing Doubling of Chromosomes in Plants by Treatment with Colchicine, *J. Heredity*, 28: 393–411 (1937).

those of the normal type. Colchicine was the only one of many chemical compounds tested which induced a doubling of the chromosome number and if Blakeslee's early observations are borne out, the geneticist will have available for the first time a chemical method of inducing directed genetic changes.

Another compound, which may be considered as intermediate between the true alkaloids and other compounds containing a basic nitrogen group, is *solanine*. Solanine occurs in the potato, and on acid hydrolysis is reported to yield one molecule, each, of dextrose, galactose, and rhamnose, and a base, *solanidine* ($C_{27}H_{43}ON$). Solanine has been reinvestigated [52] by the dehydrogenation of solanidine from potatoes. A hydrocarbon, methylcyclopentenophenanthrene or Diels hydrocarbon, was obtained, indicating that solanidine contains in part the same nucleus which is characteristic of the sterols, the sex hormones, and the digitalis glycosides and saponins (*vide infra*). On the basis of the reinvestigation the probable formula of solanidine is:



Solanidine (Soltys, 1936)

Solanine is, therefore, possibly an alkaloidal glycoside. Solanine is obtained commercially from the juice of potato sprouts, and nearly every year workers in agricultural experiment stations receive reports of the deaths of farm animals which on checking back are found to be due to the feeding either of potato sprouts or of sprouted potatoes. Under ordinary conditions, potatoes seldom contain enough to produce toxic effects, but certain strains of potatoes contain appreciable amounts. "Sunburned" potatoes are nearly always bitter and contain an increased quantity of solanine. Death may result, if a considerable quantity of such tubers are eaten,[53] especially if they are baked and

[52] Soltys, A., and Wallenfels, K., Über das Solanin und Solanidin, *Ber.*, 69B: 811–818 (1936).

[53] Bömer, A., and Mattis, H., Über höhe Solaningehalte bei Kartoffeln, *Z. Nahr. Genussm.*, 45: 288–291 (1923); Der Solaningehalt der Kartoffeln, *Z. Nahr, Genussm.*, 47: 97–127 (1924); *cf.* also Rothe, J. C., Über Erkrankungen nach Genuss von Solaninhaltigen Kartoffeln, *Z. Hyg. Infektionskrankh.*, 88: 1–12 (1919); Behre, A., and Ehrecke, H., Solaningehalt von Kartoffeln, *Chem. Ztg.* 42: 593 (1918).

eaten "in their jackets." Potatoes normally contain about 0.024 part of solanine per 1,000, but sunburned potatoes have been found to contain as high as 0.588 part per 1,000. Approximately 70 per cent of the solanine is removed in the parings.

The Origin of Alkaloids in Plants.—Obviously the question of origin and purpose of such compounds as the alkaloids, in the economy of a plant, is a question which will probably always remain a subject of speculation. Inasmuch as they do not occur in all forms of plant life, it is obvious that they are not essential to the life process of a plant. The great variability in amount from season to season and from locality to locality also indicates that they may possibly be regarded as a by-product of the synthetic and metabolic activities of the plant. This is the view of Pictet, who believes that the nitrogen residues from protein utilized by the plant in its metabolic processes are resynthesized into alkaloids (by certain plants which possess such synthetic power), because in this form the alkaloids are less harmful to the plant than the direct decomposition products of the proteins. Plants do not excrete their waste products, so do not synthesize any considerable quantities of urea or uric acid. The above view is obviously only a guess. We have no evidence as to the real significance of the alkaloids in the organs or tissues of a plant. It has been suggested that they are a form of protection, so that animals will not eat them, or if they do eat them, they will be "punished" and die. Such a view, however, attributes rather too much intelligence to a plant!

The alkaloidal content may be increased or decreased by appropriate selection of strains, by plant breeding, and by fertilizing (especially nitrogen and phosphorus). Collecting at the proper time is also an important factor. Thus, the first lancings of the poppy capsules yield an opium containing a very much greater morphine content than the later lancings.[54]

The nicotine content in tobacco has been increased by plant breeding and manuring. This is desirable in certain instances in order to secure a high nicotine content for spray manufacture.

[54] Annett, H. E., Factors Influencing Alkaloidal Content and Yield of Latex in the Opium Poppy (*Papaver somniferum*), *Biochem. J.*, 14: 618–636 (1920); Investigations on Indian Opium, No. 2. The Effect of Environmental Factors on the Alkaloidal Content and Yield of Latex from the Opium Poppy (*Papaver somniferum*) and the Bearing of the Work on the Functions of Alkaloids in Plant Life, *Mem. Dept. Agr. India, Chem. Series*, 6: 61–154 (1921); (and Singh, H. D.) Investigations on Indian Opium, No. 4. Further Experiments on the Influence of Manures on the Yield and Morphine Content of the Latex from the Opium Poppy, *ibid.*, 8: 27–37 (1925).

III

CARBOHYDRATES

"Für das Studium der chemischen Prozesse im Tier- und Pflanzenkörper ist nächst den Eiweisskörpern keine Gruppe von Kohlenstoffverbindungen so wichtig, wie die Kohlenhydrate, und als Nahrungsmittel nehmen sie unstreitig die erste Stelle ein."

EMIL FISCHER (1890)

CHAPTER XXII

GENERAL CONSIDERATIONS—THE SYNTHESIS OF CARBOHYDRATES IN NATURE AND THE CLASSIFICATION OF CARBOHYDRATES

Optical Rotation.—For our purposes, light may be defined as "radiant energy, propagated in free space with the velocity common to all radiant energy but having wave lengths such that it affects the human eye." It is obvious that the vibrations comprising light may take place in all directions in a plane perpendicular to the path of the beam of light. However, certain minerals, such as tourmaline, transmit light through the crystal but allow it to pass only in a definite plane, *i.e.*, perpendicular to the laminae of the crystal, so that light vibrating in only a single plane can pass through the crystal. The light which does pass is *plane polarized light*, although it is commonly referred to simply as *polarized light*.

The action of a polarimeter may be illustrated by means of two books and a ruler. If the two books are lying end to end on a table, a ruler may pass between the leaves of both and project at each end. Here, there is no resistance to the ray of light passing through the two crystals. If, however, one of the books is rotated so that the planes of the leaves do not coincide with the planes of the leaves of the other book, the ruler can no longer be passed through both volumes without being bent or twisted, and the ruler must be twisted through the same angle as that through which the book was rotated.

Biot, in 1815, discovered that certain substances, such as turpentine (liquid) or a solution of sugars or tartaric acid, *rotated* the plane of polarized light. The angle of rotation is determined by use of the polarimeter which contains two prisms of tourmaline in a form known as "Nicol prisms." These prisms are set in such a manner that light readily passes through both prisms from the light source to the eye of the observer. Then a cylinder containing the liquid under investigation is placed between the two Nicol prisms, and if the solution rotates the plane of polarized light, the field becomes black. The second Nicol prism is now rotated until light again passes through, and the number of degrees through which the prism was rotated is read from the scale.

In making studies of optical rotation it is very desirable to use monochromatic light, *i.e.*, light of a single wave length. Ordinarily the sodium light is used, inasmuch as sodium light is largely that of the D

sodium line in the yellow part of the spectrum. The mercury-vapor lamp, however, is coming into rather general use. Here we are dealing with several lines close together in the green portion of the spectrum.

The optical rotation of a substance is usually expressed as the *specific rotation* which is defined as *the number of degrees of angular rotation produced in a 1-decimeter tube containing a solution of 1 gram of substance in 1-cc. volume.*

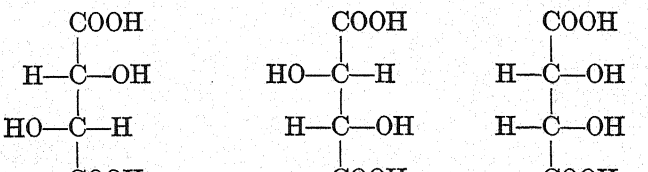
Optical Isomerism.—Certain substances possess the physical properties of optical activity when they are (*a*) in the crystalline state, *e.g.*, quartz, (*b*) in solution, *e.g.*, sugars, or (*c*) in the liquid form, *e.g.*, lactic acid, turpentine, etc.

Pasteur discovered that optical activity occurred in more than one form, *e.g.*, that tartaric acid could exist in both *dextro* and *levo* modifications and that the one rotated the plane of polarized light to the right and the other rotated it to the left. He also showed that racemic acid was an equal mixture of the *d*- and *l*-forms.

Van't Hoff (1874), and Le Bel (1874) independently advanced the theory of an asymmetric carbon atom where four different groups are attached to the same carbon atom.



Here we have two substances which are mirror images of each other and yet are structurally different. Thus, for tartaric acid we have the three possibilities,



and additional evidence for these structures is that we can secure tartaric acid from maleic and fumaric acids by oxidation with potassium permanganate. Maleic and fumaric acids differ only in their space configurations.



Maleic acid yields only *meso*-tartaric acid. Fumaric acid yields a racemic mixture of *d*- and *l*-tartaric acids. *meso*-Tartaric acid is *internally compensated*, having in the formula a top carbon the same as in the *d*-form and a bottom carbon of the *l*-form. Consequently one carbon is *d* and the other is *l*, and the rotations *within the molecule* neutralize each other, so that no rotation of the light results. Naturally occurring tartaric acid is the *d*-form.

When, as in the carbohydrates, the chemical groupings at the opposite ends of the carbon chain are different, the number of possible isomers and of racemic mixtures due to asymmetric carbon atoms can be calculated from the equations:

$$N = 2^n \tag{181}$$

and

$$r = \frac{N}{2} \tag{182}$$

where N = number of isomers;
n = number of asymmetric carbon atoms;
r = number of racemic mixtures.

The following table shows that, as the number of asymmetric carbon atoms in such compounds increases, there is a very great increase in the number of possible isomeric forms.

n =	1	2	3	4	5	6	7	8
N =	2	4	8	16	32	64	128	256
r =	1	2	4	8	16	32	64	128

In the more recent formulas for the carbohydrates we have *five* asymmetric carbon atoms in a C_6 sugar. Consequently 32 C_6 sugars are possible, *all of which will have exactly the same chemical composition.*

Carbohydrates are remarkable because of their optical activity. Using the free —CHO formula, there are 16 possible isomeric aldohexoses, $C_6H_{12}O_6$. *Only 4 occur in nature, d-glucose, d-mannose,* and *d-* and *l-galactose.*[1] This natural limitation of the number of hexoses produced by the plant or utilized by either the plant or animal kingdom is extremely interesting, as indicating a selective process during the period of organic evolution. It also indicates a rather remarkable unanimity in the evolutionary process as applied to nature as a whole.

It should be noted at this point that the prefixes, *d* and *l* (*e.g.*, *d*-mannose $[\alpha]_D$ $-14.6°$, *d*-fructose $[\alpha]_D^{20}$ $-93.5°$, and *d*-glucose $[\alpha]_D^{20}$ $+52.5°$), have entirely different meanings *so far as sugar chem-*

[1] *l*-Galactose has been isolated in quantity from flax-seed mucilage (*cf.*, Anderson, E., The Preparation of *l*-Galactose from Flax-seed Mucilage, *J. Biol. Chem.*, 100: 249–253 (1933).

istry is concerned from their meanings in other fields of chemistry. In all other branches of chemistry they refer to the direction of the rotation of the plane of polarized light, *i.e.*, whether the rotation is clockwise or counter-clockwise. This is not necessarily true for the carbohydrates. *A d-sugar is a sugar which is structurally related, so far as the asymmetry of its carbon atoms is concerned, to d-glucose. An l-sugar is similarly structurally related to l-glucose.* Accordingly a number of the known sugars may belong to the *d*-series and still may rotate the plane of polarized light counter-clockwise. Similarly certain of the *l*-sugars may have a pronounced *dextro* rotation. Therefore, *d* and *l* as prefixes to the names of the carbohydrates signify only their *family relationships* and bear no necessary reference to their optical rotation, excepting in the case of *d*- and *l*-glucose, to which all the others are referred.

This nomenclature was introduced by Emil Fischer during his studies of the synthesis of carbohydrates. It is without doubt a confusing element in carbohydrate study. Nevertheless, this particular nomenclature, so far as the carbohydrates are concerned, is so firmly fixed in the literature that it seems impracticable to alter it. It is to be regretted that Fischer did not use some other symbols to designate structural relationships.

THE IMPORTANCE OF CARBOHYDRATES.—Carbohydrates and proteins play a major role in both plant and animal life. *The carbohydrates are the fuel which serves to carry on the vital processes.*

Interest in carbohydrates naturally centers around glucose, for this is one of the most important of the sugars, probably the most important. Whether or not it is the primary product of carbohydrate synthesis in the plant is still uncertain. It is nevertheless the unit from which are derived sucrose, maltose, starch, glycogen, and cellulose. The bulk of carbohydrates in our body metabolism is transported as glucose, and in the form of glucose the plant transports its carbohydrates.

The carbohydrates as a group constitute three-fourths of the dry weight of the plant world. Something like 40 sugars have been isolated from plant or animal sources.

THE SYNTHESIS OF CARBOHYDRATES IN NATURE.—In the higher plants, the carbohydrates are produced through photosynthesis, and since most animals depend for their energy upon the higher plants, the higher forms of animal life are likewise dependent upon the photosynthetic process.

The synthesis of carbohydrates in nature, however, is not wholly through a photosynthetic mechanism. Many bacteria can synthesize protoplasm *in the dark* from wholly inorganic elements. A protozoan, *Chilomonas paramecium*, can grow and multiply in a purely inorganic medium where only the inorganic forms of N, C, O, H, S, P, K, and Mg

are present in appreciable amounts. *It can synthesize fats and starch* [2] *when CO_2 is the sole source of carbon and can do this equally as well in the dark as in the light.* The nature of the mechanism is unknown.

The mode of synthesis of carbohydrates in the plant is still uncertain. We know that in the presence of sunlight and chlorophyll, under the conditions which are present in green leaves, carbon dioxide and water unite to form carbohydrates, and that oxygen is evolved during the synthesis. This particular chemical reaction is without doubt the most important chemical reaction taking place on the earth, for by this reaction both plants and animals have made available to them a source of energy.

One cannot emphasize too strongly that in the plant the synthesis of all organic substances must revert to the initial mechanism involved in the synthesis of carbohydrates, so that the synthesis of proteins, fats, alkaloids, acids, etc., is in the last analysis dependent upon the photosynthetic mechanism.

Numerous theories [3] have been proposed to explain the mechanism which takes place. We will only mention in a casual way two of the more important of the theories.

The Formic Acid Theory.—This theory assumes that formic acid is the initial product of photosynthesis, the carbon dioxide undergoing reduction to formic acid with the formation of hydrogen peroxide, the formic acid being then transposed into carbohydrates,

$$CO_2 + 2H_2O = H—COOH + H_2O_2$$

Although this theory has received some support in recent years, it must still remain a theory without experimental proof.

The Formaldehyde Theory.—The formaldehyde theory is perhaps the oldest of the theories concerning the mechanism of the process, appears the most plausible, and has attracted the attention of the most investigators. Butlerow, in 1861, observed that trioxymethylene in the presence of alkali was polymerized to a sugar (formose), $6HCHO \longrightarrow C_6H_{12}O_6$. From this sugar Fischer (*vide infra*) later synthesized the naturally occurring sugars. Baeyer,[4] in 1864, put forth the hypothesis that formaldehyde in solution was in reality

[2] Mast, S. O., and Pace, D. M., Synthesis from Inorganic Compounds of Starch, Fats, Proteins, and Protoplasm in the Colorless Animal, *Chilomonas paramecium, Protoplasma*, 20: 326–358 (1933).

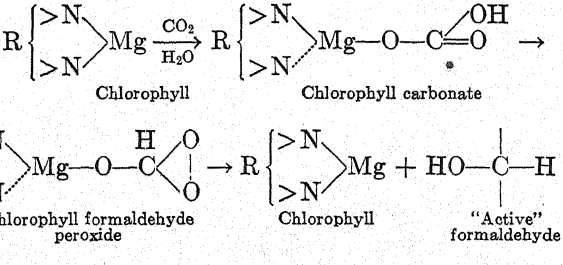
[3] Spoehr, H. A., Photosynthesis, Chemical Catalog Company, New York (1926); *cf.* also Meldola, R., The Living Organism as a Chemical Agency; A Review of Some of the Problems of Photosynthesis by Growing Plants, *J. Chem. Soc.*, 89: 749–770 (1906); Willstätter, R., and Stoll, A., Untersuchungen über die Assimilation der Kohlensäure, Julius Springer, Berlin (1918); Kostychev, S. P., Plant Respiration (translated by Charles J. Lyon), P. Blakiston's Son and Company, Philadelphia (1927).

[4] Baeyer, A., Ueber die Wasserentziehung und ihre Bedeutung für das Pflanzenleben und die Gährung, *Ber.*, 3: 63–75 (1870).

methylene glycol, $CH_2(OH)_2$, and that Butlerow's reaction should be written $6CH_2(OH)_2 = C_6H_{12}O_6 + 6H_2O$. Baeyer believed that formaldehyde was the initial product in photosynthesis, that the carbon dioxide was first reduced to carbon monoxide and oxygen, and that carbon monoxide was then reduced to formaldehyde, which then condensed to a sugar, possibly through the intermediate stages of glycolic aldehyde or glyceric aldehyde. This hypothesis has interested investigators along three different lines: (1) the reduction of carbon dioxide in aqueous solution to formaldehyde by chemical and photochemical means, (2) a search for formaldehyde or other intermediate products in green plants during active photosynthesis, and (3) formaldehyde as a source of energy for plant life. Researches along all three of these lines have yielded valuable results, the only question at issue being the interpretation of *in vitro* experiments to *in vivo* action.

Fenton,[5] in 1907, showed that carbon dioxide could be reduced by appropriate means to formaldehyde. Within recent years the use of catalysts has confirmed this observation. However, the conditions existing in the plant are so different from the conditions used for the industrial reduction of carbon dioxide that the question arises whether the plant possesses the mechanism for the production of formaldehyde in this manner.

Willstätter and Stoll suggest the following sequence of reactions in which first the magnesium in the chlorophyll molecule (*vide infra*) combines with carbon dioxide and water to form a chlorophyll carbonate, then this rearranges to form a chlorophyll formaldehyde peroxide, and this breaks down to regenerate chlorophyll and to yield oxygen and an active form of formaldehyde which immediately polymerizes to a carbohydrate, probably without going through the aldehydic form of formaldehyde.



Hibbert[6] suggests ethylene oxide as an intermediate compound in the synthesis of carbohydrates in nature.

[5] Fenton, H. J. H., The Reduction of Carbon Dioxide to Formaldehyde in Aqueous Solution, *J. Chem. Soc.*, 91: 687–693 (1907).

[6] Hibbert, H., and Perry, S. Z., The Nature of the Mechanism of Polymerization, *Can. J. Research*, 8: 102 (1933); Hibbert, H., The Plant Synthesis of Carbohydrates and Polysaccharides, *ibid.*, 8: 103–104 (1933).

Numerous workers have announced from time to time their findings of formaldehyde in green leaves, only to have the evidence overthrown by later and more accurate investigations. Klein and Werner,[7] however, report formaldehyde as a constant product in leaves undergoing active photosynthesis. They apparently eliminated the possibility that the formaldehyde which they obtained arose as a decomposition product of the organic materials present. They made use of the new reagent, "dimedon" (dimethylhydroresorcinol), which condenses with formaldehyde to form an extremely insoluble and characteristic crystallization product. They observed the presence of formaldehyde regularly in leaves undergoing photosynthesis, whereas leaves which had been exposed to the dark for some time contained no formaldehyde, nor did chlorophyll extracts or leaves which had been macerated. Similarly, in experiments where leaves were exposed to the light in a carbon dioxide-free atmosphere, no formaldehyde was produced. Neither was it produced when leaves were treated with narcotics or poisons, such as phenylurethan, hydrocyanic acid, or sulfurous acid. They note that the concentration of formaldehyde remains at a very low level (8 to 15 mg. per 10 grams of fresh leaves), which would indicate that, if it is an intermediate product of photosynthesis, it is very rapidly condensed into sugars.

Numerous investigators have studied solutions of formaldehyde and other aldehydes as a source of energy for plant life. Sabalitschka and Weidling[8] note that *Elodea canadensis* can utilize formaldehyde in the dark, converting it into starch. Illumination does not appreciably alter this process. A concentration of 0.024 per cent formaldehyde gave the best results, more than that amount producing a toxic effect. The fact that it is utilized in the dark and transformed into starch is taken as evidence that it is a normal product of photosynthesis.

In a later paper[9] the same authors report studies on the assimilation of acetaldehyde by *Elodea canadensis*, and they find again that acetaldehyde is transformed into starch by the plants grown both in the dark and in the light. Figure 140 shows their results expressed in percentage of starch in 100 grams of dry material after the plants have been kept in the dark for a period of 10 days in aldehyde solutions of different concentrations. We must conclude that both formaldehyde and acetaldehyde may act as nutrients for green plants, both being

[7] Klein, G., and Werner, O., Formaldehyd als Zwischenprodukt bei der Kohlensäureassimilation, *Biochem. Z.*, 168: 361–386 (1926).

[8] Sabalitschka, Th., and Weidling, H., Über die Ernährung von Pflanzen mit Aldehyden. VI. Polymerisation des Formaldehyds durch Elodea canadensis zu höheren Kohlehydraten, *Biochem. Z.*, 172: 45–57 (1926).

[9] Sabalitschka, Th., and Weidling, H., Über die Ernährung von Pflanzen mit Aldehyden. VII. Erhöhung des Kohlehydratgehaltes von Elodea canadensis durch Acetaldehyd, *Biochem. Z.*, 176: 210–224 (1926).

capable of being transformed into starch and stored in the leaves, or of being used as energy sources by the growing plant.

WHAT IS THE FIRST SUGAR FORMED IN THE PROCESS OF PHOTOSYNTHESIS?—Spoehr considers this problem at length and notes that the evidence is still inconclusive. The solution of this problem would greatly assist us in answering the questions as to the mechanism of photosynthesis. The data which are available, however, are very conflicting. Thus, Brown and Morris,[10] from a study of the garden nasturtium, came to the conclusion that sucrose is the first product of photosynthesis, that it is a temporary reserve material which accumulates during the day, and that when it reaches a certain concentration, it is converted into starch. For translocation it is hydrolyzed into

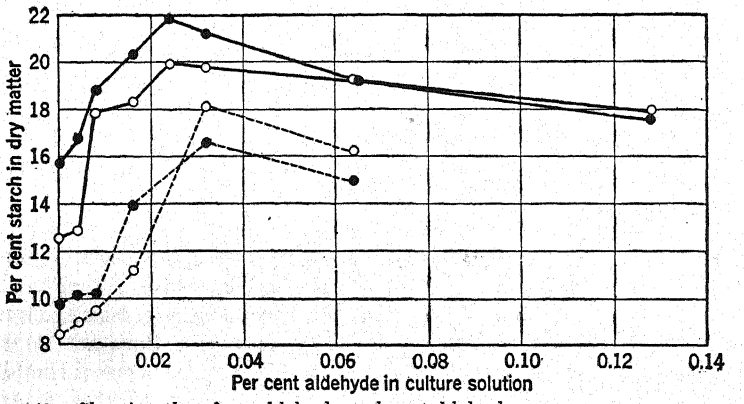


FIG. 140.—Showing that formaldehyde and acetaldehyde may serve as a source for starch formation by *Elodea canadensis*. Solid lines show formaldehyde; dotted lines, acetaldehyde data. Open circles, 10 days in the light; solid dots, 10 days in the dark. (Data of Sabalitschka.)

glucose and fructose. The fact that leaves of the nasturtium which have been actively photosynthesizing contain no glucose or fructose is the basis for their argument that these sugars cannot be the primary products. On the other hand, Priestley,[11] from a somewhat similar study, reaches the conclusion that sucrose is not the first sugar which is formed, and he concludes with the statement that the question must be regarded as still unsolved. Spoehr concludes his discussion of this subject with the statement (p. 220), "In the present state of our knowledge glucose fits the theoretical requirements most adequately. Yet the fact cannot be entirely disregarded that the demonstration of glucose actually being the first sugar formed is still wanting."

[10] Brown, H. T., and Morris, G. H., A Contribution to the Chemistry and Physiology of Foliage Leaves, *J. Chem. Soc.*, 63: 604–677 (1893).

[11] Priestley, J. H., The First Sugar of Photosynthesis and the Rôle of Cane Sugar in the Plant, *New Phytologist*, 23: 255–265 (1924).

THE ENERGY RELATIONS OF PHOTOSYNTHESIS.—The two fundamental equations of living processes from the energy standpoint are usually written as,

$$6CO_2 + 6H_2O = C_6H_{12}O_6 + 6O_2 \text{ (photosynthesis)}$$

and

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O \text{ (respiration)}$$

In writing these chemical equations, the most significant part of the equations, so far as vital processes are concerned, has been omitted, *i.e., the energy relationships.* The equations when correctly written should be

$$6CO_2 + 6H_2O + 677.2 \text{ Cal.} = C_6H_{12}O_6 + 6O_2 \text{ (photosynthesis)}$$

and

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O + 677.2 \text{ Cal. (respiration)}$$

It is through photosynthesis that the energy of sunlight is fixed and through oxidation that this fixed energy is released for the vital processes.

It is generally stated that the efficiency of photosynthesis is extremely low. Warburg and Negelein [12] note that the efficiency of photosynthesis for the alga, *Chlorella*, ranges from 59 to 63.5 per cent on the hypothesis that glucose is the primary product of photosynthesis. In this instance no energy was required for transpiration, since the algae are surrounded by water. Adams [13] suggests that perhaps the reaction may be written

$$6[CO_2 + 3H_2O = 2H_2O_2 + HCHO]$$

and on this basis, accounting for the energy in the formation of hydrogen peroxide, he calculates an efficiency of 98.6 per cent of the absorbed light.

Transeau [14] has summarized the energy relationships in a very striking manner. He selected an acre of corn in environmental conditions of north central Illinois and prepared a "budget" of the energy of the sunlight falling on that acre during the growing period and the fixation of the energy by the corn plant.

Taking a growing season of 100 days with 10,000 corn plants to the acre, and assuming a yield of corn of 100 bushels with a dry weight

[12] Warburg, O., and Negelein, E., Über den Einfluss der Wellenlänge auf den Energieumsatz bei der Kohlensäureassimilation, *Z. physik. Chem.*, 106: 191–218 (1923).

[13] Adams, E. Q., The Efficiency of Photosynthesis by *Chlorella, J. Am. Chem. Soc.*, 48: 292–294 (1926).

[14] Transeau, E. N., The Accumulation of Energy by Plants, *Ohio J. Sci.*, 26: 1-10 (1926).

of 2,160 kg. per acre, the total dry weight of the 10,000 plants at maturity is approximately 6,000 kg., 322 kg. of which is mineral matter, leaving a residue of 5,678 kg. of organic matter of which 2,675 kg. is carbon. This amount of carbon would be equivalent to 6,687 kg. of glucose. This then is the amount of primary sugar equivalent to the carbon accumulated by the mature plant.

Transeau then notes that at maturity only a part of the carbon formed by synthesis remains. A part has been lost by the plant in the process of respiration. He estimates respiration to release an amount of carbon dioxide per day equivalent to 1 per cent of the dry weight of the plant. The average dry weight for the season Transeau takes as one-half of the total weight, *i.e.*, 3,000 kg. There would accordingly be 3,000 kg. of carbon dioxide, equivalent to 818 kg. of carbon lost by respiration during the growing season. The glucose equivalent of the carbon dioxide respired for the entire acre, he calculates to be 2,045 kg. He then adds this amount of glucose to the glucose equivalent of the carbon in the plant at maturity, giving the total glucose manufactured as 8,732 kg. Inasmuch as it requires energy equivalent to 3,760 Calories to produce 1 kg. of glucose, he notes that it requires not far from 33,000,000 Calories to produce the entire photosynthetic product. The summation of the energy consumed in photosynthesis is shown in Table LXX.

TABLE LXX

ENERGY CONSUMED IN PHOTOSYNTHESIS
(One acre of *Zea mays*, growing season of 100 days)
(Data of Transeau)

Glucose equivalent of accumulated carbon	6687 kg.
Glucose equivalent of carbon oxidized	2045 kg.
Total glucose manufactured	8732 kg.
Energy required to produce 1 kg. glucose	3760 Cal.
Total energy consumed in photosynthesis	33 million Cal.

Transeau then proceeds to estimate the efficiency of photosynthesis, noting that the total energy of sunlight falling on an acre of ground during the growing season is approximately 2,043 million Calories. Of this enormous amount, only 1.6 per cent is accounted for in Table LXX as energy consumed in photosynthesis. He notes, however, that only approximately 20 per cent of the light of the spectrum is efficient for photosynthesis. On this basis, however, the efficiency of the photosynthetic process would be only 8 per cent. Facts such as this have been widely quoted to illustrate the inefficiency of the photosynthetic process.

Transeau, however, proceeds to note that there is another source of energy lost to the plant, *i.e.*, transpiration. Under the Illinois conditions which were studied, approximately 276 kg. of water are evaporated during the growing season for every kilogram gained in dry weight. The total weight of water lost by transpiration is, therefore, approximately 1,500,000 kg. which would be equivalent to 408,000 gallons or sufficient water to cover the acre to a depth of 15 inches. The energy required to evaporate 1 kg. of water at the average temperature of the growing season is approximately 593 Calories, or the total energy consumed in the process of transpiration is approximately 910,000,000 Calories, equal to 44.5 per cent of the total energy of the sun falling upon the acre.

Respiration again releases a part of the energy rendered potential in photosynthesis. We have already noted that 2,045 kg. of glucose are oxidized. This results in the liberation of 7,700,000 Calories, or almost one-fourth of the energy absorbed in the process of photosynthesis. Transeau notes that if we assume that photosynthesis goes on for 12 hours during the day, and respiration 24 hours each day, the average rate of photosynthesis must be about 8 times as great as the rate of respiration.

Transeau further notes that for a very considerable part of the growing season, the plants do not completely shade the ground. At the beginning of the growing season, the plants are extremely small, most of the acre is bare, and the energy falling upon this bare earth ought not to be charged against the efficiency of the photosynthetic process. It is only late in the growing season that the plants become sufficiently developed to completely shade the ground and thus intercept all of the light energy falling upon the acre.

Table LXXI shows the summary budget presented by Transeau.

TABLE LXXI

SUMMARY OF ENERGY BUDGET OF ONE ACRE OF CORN DURING GROWING SEASON

Total radiant energy available	2043 million Cal.
Used in photosynthesis	33 million Cal.
Used in transpiration	910 million Cal.
Total energy consumed	943 million Cal.
Energy not directly used by the plants	1100 million Cal.
Energy released by respiration	8 million Cal.
Of the available radiant energy, 100-bushel-corn uses about	46%
The environment takes up about	54%

It will be noted that the acre of corn utilized 46 per cent of the energy falling upon that acre, and the environment, *i.e.*, the air, the bare ground, etc., used up 54 per cent. If we assume, as seems reasonable,

that the corn plant shaded the surface of the soil completely for only one-half of the time, Transeau's budget would account for a very high efficiency of the utilization of solar energy.

The striking conclusions of Transeau's calculations are that the evaporation of water from an acre of growing corn consumes about 45 per cent of the available radiant energy, that of the carbohydrates synthesized approximately 23.4 per cent is again utilized by the plant for purposes of respiration, that the total energy content of the dry matter of the corn plant at maturity is equal to only 1.6 per cent of the energy falling upon the area during the growing period, and, if we consider only the grain, we have returned to us less than 0.5 per cent of the total radiant energy.

Transeau believes that corn is probably the most efficient of our temperate-zone plants and that very few of the crop plants in the temperate regions can equal corn in calories of energy per acre. He further points out that the suggestion has been made that when the liquid fuels, such as petroleum and gasoline, are gone, they may be replaced by alcohol made from plants, and notes that to substitute the energy of alcohol for the energy which is at the present time being used in the United States in the form of gasoline would require more corn than is now being grown in the United States.

Shull [15] has made a further contribution to the question of the energy relationships. In all the earlier calculations on the efficiency of photosynthesis, it has been assumed that the light falling upon a leaf is all potentially available. Shull measured by means of a spectrophotometer the reflection of light from the surface of leaves. The percentage of reflection was usually low in the blue and red regions and highest in the green, the curve of reflection rising to a maximum of 540 to 560 mμ. The lowest reflection was found in very dark green leaves, such as those of *Syringa vulgaris*, where the reflection was only 6 to 8 per cent, ordinary green leaves reflecting from 10 to 12 per cent. Spring verdure has a much higher reflection which may reach 20 per cent or more, while albino leaves and those which are pubescent, such as *Populus alba*, may reflect from 30 to 52 per cent of the incident light. It would seem that we should not charge the chloroplast with inefficiency in the photosynthetic mechanism when a very considerable proportion of the light never reaches the chloroplasts.

Classification of the Carbohydrates

I. Simple Sugars or Monosaccharides.—These may be defined as aldehyde or ketone alcohols of the aliphatic series, the molecule of which contains one carbonyl group and one or more alcohol groups, one of the

[15] Shull, C. A., Reflection of Light from the Surfaces of Leaves, *Science*, 67: 107–108 (1928).

latter always being adjacent to the carbonyl group. According to this definition formaldehyde is not a carbohydrate.

A. The *aldose* sugars contain a potential aldehyde group.

B. The *ketose* sugars contain a potential ketone group.

The simple sugars may be further subdivided according to the number of carbon atoms.

1. Monoses, CH_2O
   Formaldehyde, first homolog of series *but not a carbohydrate*
2. Dioses, $C_2H_4O_2$
   Glycolaldehyde
3. Trioses, $C_3H_6O_3$
   a. Aldotriose
      d- and *l*-glycerose (glyceric aldehyde)
   b. Ketotriose
      Dioxyacetone
4. Tetroses, $C_4H_8O_4$
   a. Aldotetroses (4 possible isomers)
      d- and *l*-erythrose
      d- and *l*-threose
   b. Ketotetroses
      Erythrulose
   c. Hydroxymethyltetrose
      Apiose (*β*-hydroxymethyltetrose from the glycoside, apiin)
5. Pentoses, $C_5H_{10}O_5$
   a. Aldopentoses (8 possible isomers)
      d- and *l*-arabinose
      d- and *l*-xylose
      d- and *l*-ribose
      d- and *l*-lyxose
   b. Methylpentoses (One of the hydrogen atoms of the primary alcohol group in an aldopentose is replaced by a methyl group.)
      Rhamnose (component of many vegetable glycosides)
      Fucose (Fucosan found in seaweed of Fucus variety[16])
      Rhodeose (occurs in certain glycosides)
   c. Methoxymethyl pentose
      Digitalose ($C_7H_{14}O_5$, in the glycoside, digitalin)
   d. Methylthio pentose ($C_6H_{12}O_4S$, from yeast, cf. p. 488)
   e. Ketopentoses (4 possible isomers)
      l-ketoxylose[17]

[16] Clark, E. P., The Structure of Fucose, *J. Biol. Chem.*, 54: 65–73 (1922).

[17] Levene, P. A., and LaForge, F. B., Note on a Case of Pentosuria, *J. Biol. Chem.*, 18: 319–327 (1914); *cf.* also, Greenwald, I., *J. Biol. Chem.*, 88: 1–7 (1930); 89: 501 (1930); 91: 731–734 (1931).

f. 2-Desoxypentoses
d-2-desoxyribose ($C_5H_{10}O_4$, from thymonucleic acid)
d-2-desoxymethylpentose ($C_6H_{12}O_4$, digitoxose, from digitalis glycoside)

6. Hexoses, $C_6H_{12}O_6$
a. Aldohexoses (16 possible isomers, all known; 4 occur in nature)
d- and *l*-mannose
d- and *l*-glucose
d- and *l*-idose
d- and *l*-gulose
d- and *l*-galactose
d- and *l*-talose
d- and *l*-allose
d- and *l*-altrose
b. Ketohexoses (8 possible isomers, 5 known)
d- and *l*-fructose
d- and *l*-sorbose
d-tagatose
c. Aminohexoses
Chitosamine or glucosamine (2-aminoglucose)
Chondrosamine (2-aminogalactose)

7. Heptoses, $C_7H_{14}O_7$
a. Aldoheptoses (none naturally occurring, those known being synthetic)
Glucoheptose
Mannoheptose
Galactoheptose
b. Ketoheptoses (2 naturally occurring)
Sedoheptose (*d*-altroheptulose[18])
d-mannoketoheptose (from the avocado,[19] *Persea gratissima*)
d-glucoheptulose (synthetic)
l-glucoheptulose (*Bacterium xylinum* action on α-glucoheptitol)
Perseulose (*l*-galactoheptulose by *Bacterium xylinum* action on α-mannoheptitol from seeds of avocado)

8. Octoses, $C_8H_{16}O_8$
a. Aldooctoses (none naturally occurring)
Glucoöctose
Mannoöctose
Galactoöctose

[18] La Forge, F. B., and Hudson, C. S., Sedoheptose, A New Sugar from *Sedum spectabile*. I., *J. Biol. Chem.*, 30: 61–77 (1917).

[19] La Forge, F. B., *d*-Mannoketoheptose, A New Sugar from the Avocado, *J. Biol. Chem.*, 28: 511–522 (1917).

9. Nonoses, $C_9H_{18}O_9$
   a. Aldononoses (none naturally occurring)
      Gluconononose
      Mannononose

10. Decoses, $C_{10}H_{20}O_{10}$
   a. Aldodecoses (none naturally occurring)
      Glucodecose

The pentose and hexose sugars are the most important of the simple sugars.

II. *Compound Sugars.*—By the condensation of 2, 3, or 4 molecules of the monosaccharides, disaccharides, trisaccharides, and tetrasaccharides are formed. In these condensations one molecule less of water is eliminated than the number of reacting sugars.

$$2C_6H_{12}O_6 - H_2O = C_{12}H_{22}O_{11} \text{ (disaccharide)}$$
$$3C_6H_{12}O_6 - 2H_2O = C_{18}H_{32}O_{16} \text{ (trisaccharide)}$$
$$4C_6H_{12}O_6 - 3H_2O = C_{24}H_{42}O_{21} \text{ (tetrasaccharide)}$$

1. Disaccharides
   a. Dipentose saccharides, $C_5H_9O_4—O—C_5H_9O_4$
      Diarabinose, $C_{10}H_{18}O_9$
   b. Pentose-hexose saccharides, $C_5H_9O_4—O—C_6H_{11}O_5$
      Glucoapiose, $C_{11}H_{20}O_{10}$ (in the glycoside, apiin)
      Galactoarabinose, $C_{11}H_{20}O_{10}$ (synthetic)
      Glucose-arabinoside
         Vicianose (glucose-6-*β*-*l*-arabinoside, in *Vicia angustifolia*)
      Glucose xyloside
         Primeverose (glucose-6-*β*-*d*-xyloside, in *Primula officinalis*)
   c. Methylpentose-hexose saccharides,

$$CH_3—C_5H_8O_4—O—C_6H_{11}O_5$$

      Glycorhamnoside (in the glycoside, convolvulin)
      Rutinose (a glucorhamnose occurring in rutin)[20]

   d. Dihexose saccharides, $C_6H_{11}O_5—O—C_6H_{11}O_5$ or $C_{12}H_{22}O_{11}$
      Sucrose, maltose, and lactose are the most important of the higher saccharides.

[20] Charaux, M. C., Sur la présence de la rutine dans certains végétaux. Préparation et identification de ce glucoside et de ses produits de dédoublement, *Bull. soc. chim. biol.*, 6: 641–647 (1924); *cf.* also Rabate, M. J., Sur le présence du rutoside dans les tiges foliées du "Buplevrum falcatum" L., *ibid.*, 12: 974–977 (1930).

Type I. Aldehyde group potentially functional

Maltose	Glucopyranose-4-α-glucopyranoside
Lactose	Glucopyranose-4-β-galactopyranoside
Isolactose	Glucose-?-galactoside
Melibiose	Glucopyranose-6-α-galactopyranoside
Turanose	β-Fructofuranose-6-α-glucopyranoside
Gentiobiose	Glucopyranose-6-β-glucopyranoside
Cellobiose	Glucopyranose-4-β-glucopyranoside

Type II. Aldehyde group not functional

(no reducing properties)

Sucrose	α-Glucopyranose-1-β-fructofuranoside
Trehalose	α-Glucopyranose-1-α-glucopyranoside
Isotrehalose	β-Glucose-β-glucoside

2. Trisaccharides, $C_{18}H_{32}O_{16}$
   - Type I. Reducing
     - Methylpentose-hexose saccharides
       - Rhamninose (galactose-rhamnose-rhamnoside)
       - Robinose [galactose + rhamnose + rhamnose (from the glycoside, robinine)]
     - Trihexose saccharide
       - Mannotriose (glucose-6 $\longleftrightarrow$ 1-galactose-6 $\longleftrightarrow$ 1-galactoside)
   - Type II. Non-reducing
     - Trihexose saccharides
       - Raffinose (2-β-Fructofuranose-1-α-glucose-6-α-galactoside)
       - Melezitose (1-α-Glucose-2-β-fructofuranose-6-α-glucoside)
       - Gentianose (2-β-Fructofuranose-1-α-glucose-6-β-glucoside)

3. Tetrasaccharides, $C_{24}H_{42}O_{21}$
   - Type II. Non-Reducing
     - Tetrahexose saccharides
       - Stachyose (fructofuranose-glucose-galactose-galactoside)

4. Polysaccharides or non-sugars.—Formed by the condensation of an indefinite number of monosaccharide molecules
   - *a.* Pentosans $(C_5H_8O_4)_x$
     - Araban
     - Xylan
   - *b.* Methyl pentosans
     - Rhamnan

c. Hexosans $(C_6H_{10}O_5)_x$
- Dextrosans
  - Dextrin
  - Starch
  - True or normal cellulose
  - Lichenin (a constituent of many mosses and lichens)
  - Dextran (a mucilaginous substance secreted by many bacteria)
  - Glycogen
- Levulosans
  - Inulin
- Mannosans
  - Mannan
- Galactosans
  - Galactan

d. Condensed aminosaccharides
- Aminoglucosans
  - Mucosin
  - Chitin
- Aminogalactosans
  - Chondroitin

e. Mixed pentosans
- Gums
- Mucilages
- Hemicelluloses
- Pectins

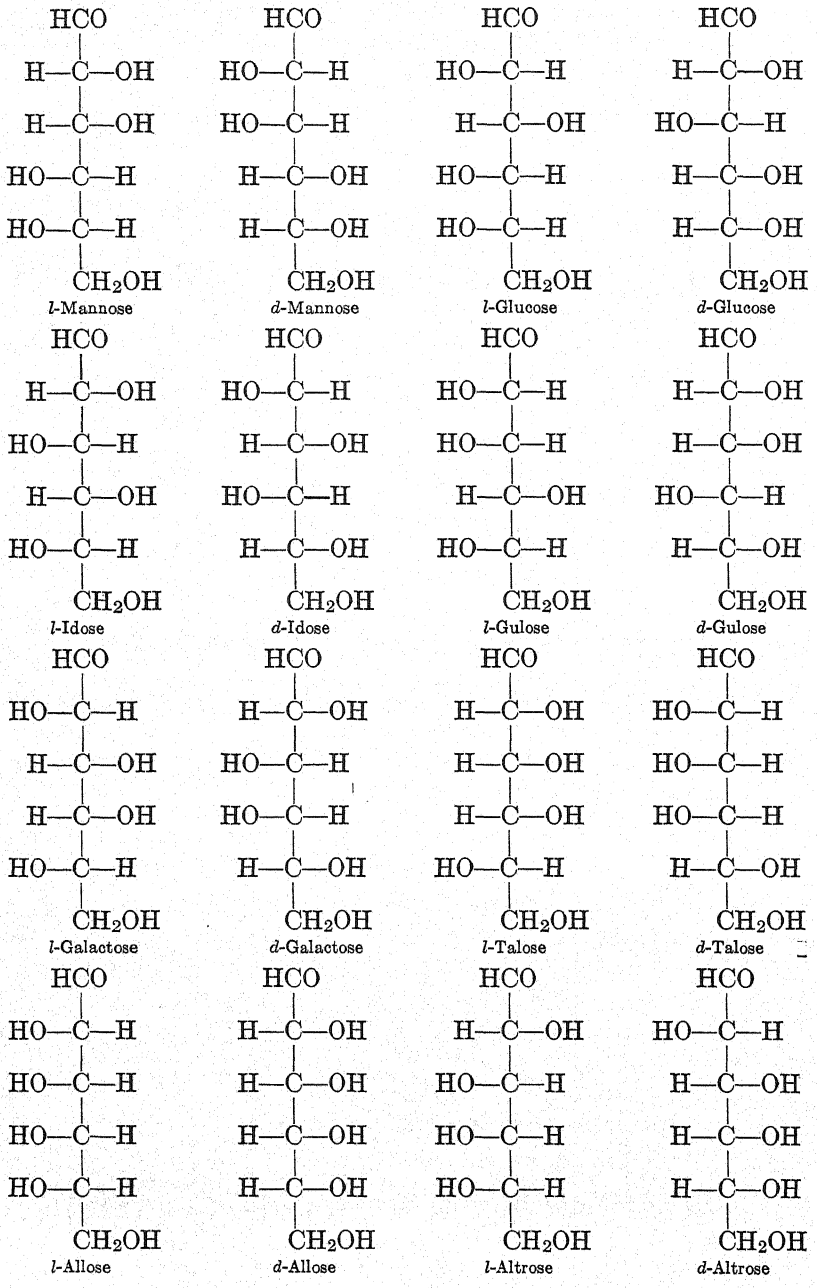
(*f*) Mixed hexosans
- Compound celluloses
  - Lignocelluloses (non-cellulose constituent-lignin)
  - Pectocellulose (non-cellulose constituents—pectic substances)
  - Adipocellulose (non-cellulose constituents—fatty substances, as suberin and cutin)

III. *The Cycloses or Cyclitols.*—These are the hexahydrohexahydroxybenzenes $(C_6H_{12}O_6)$ or closely related compounds.

1. Inositols
   - *d*-inositol
   - *l*-inositol
   - *i*(*meso*)-inositol (dambose, nucite)
   - *i*(*meso*)-inositol (scyllitol)
2. Quercitols, $C_6H_7(OH)_5$
   - *d*-quercitol
   - *l*-quercitol
3. Tetritols, $C_6H_8(OH)_4$
   - Betitol

Structural Formulas

Aldohexoses



Rosanoff,[21] in 1906, published a diagram showing the structural relationships of the various aldo sugars. This was later [22] revised to include all the aldomonosaccharides which were known in 1922. In the latter diagram, Fischer's classification was rigidly adhered to, although it contained errors in the *d*- and *l*-forms of threose, xylose, gulose, and idose. Figure 141 shows a modified diagram based on that

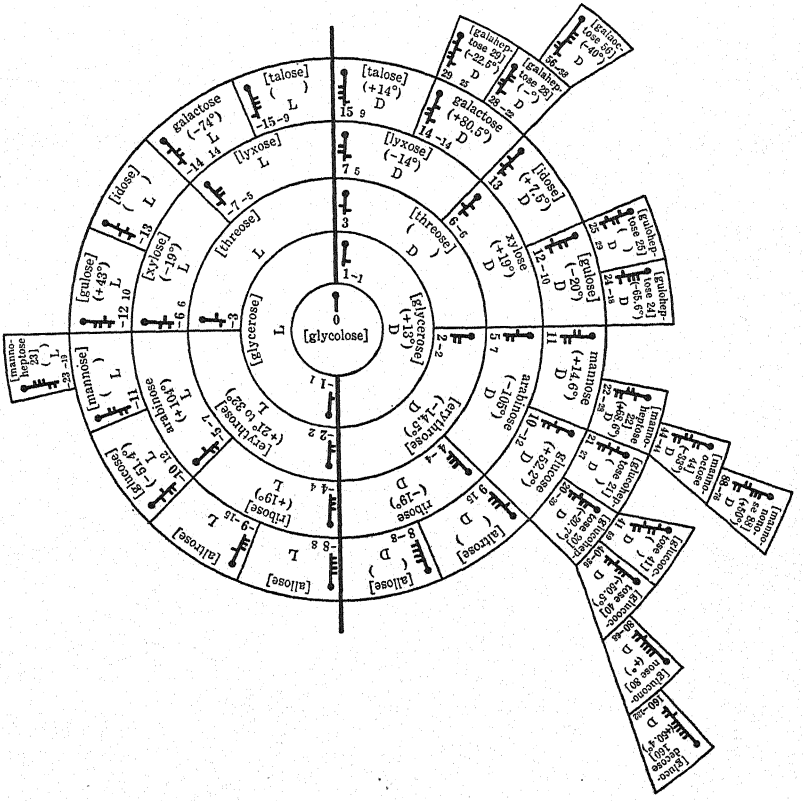


FIG. 141.—The structural relationships of the aldose sugars, together with data concerning their specific rotation. Sugars in brackets do not occur in nature. (After Rosanoff-Willaman-Morrow)

of Willaman and Morrow, excepting that the generally accepted relationships of the *d*- and *l*-forms of the four sugars noted above have been introduced into the diagram. In the diagram all the *d*-aldoses are in the right semicircle, all the *l*-aldoses in the left semicircle, and

[21] Rosanoff, M. A., On Fischer's Classification of Stereo-isomers, *J. Am. Chem. Soc.*, 28: 114–121 (1906).

[22] Willaman, J. J., and Morrow, C. A., A Revision of Rosanoff's Diagram of the Aldose Sugars, *J. Am. Chem. Soc.*, 45: 1273–1280 (1923).

the sugars which arise by the cyanhydrin reaction are placed in the next concentric circle above the parent sugar from which they are derived.

Willaman and Morrow likewise collected information regarding the alcohol and dicarboxylic acid derivatives of the aldo sugars. Their table, modified to agree with Fig. 141, is reproduced in Table LXXII.

TABLE LXXII

ALCOHOL AND DICARBOXYLIC ACID DERIVATIVES OF THE ALDOSE SUGARS

Sugars	Derivatives	
	Dicarboxylic Acids	Alcohols
Glycolose	Oxalic	Ethylene glycol
Glycerose	Tartronic	Glycerol
Erythrose	Mesotartaric	Meso-erythrol
Threose	Tartaric	Erythrol
Ribose	Ribotrihydroxyglutaric	Adonitol
Arabinose	Arabotrihydroxyglutaric	Arabitol
Xylose	Xylotrihydroxyglutaric	Xylitol
Lyxose	Arabotrihydroxyglutaric	Arabitol
Allose	Allomucic	(Allitol)
Altrose	Talomucic	Talitol
Glucose	Saccharic	Sorbitol
Mannose	Mannosaccharic	Mannitol
Gulose	Saccharic	Sorbitol
Idose	Idosaccharic	Iditol
Galactose	Mucic	Dulcitol
Talose	Talomucic	Talitol
Glucoheptose	Glucoheptonic	Glucoheptitol
Mannoheptose 22	Mannoheptonic	Perseitol
Mannoheptose 23	Mannoheptonic	Mannoheptitol
Guloheptose 24–25	Guloheptonic	Guloheptitol
Galaheptose 28	Galaheptonic	Galaheptitol
Galaheptose 29	Guloheptonic	Guloheptitol
Glucoöctose 40–41	(Glucoöctonic)	Glucoöctitol
Mannoöctose 44	(Mannoöctonic)	Mannoöctitol
Galaoctose 56	(Galaoctonic)	Galaoctitol
Gluconononose 80	(Gluconononic)	Gluconononitol
Mannononose 88	(Mannononic)	(Mannononitol)
Glucodecose 160	(Glucodeconic)	Glucodecitol

CHAPTER XXIII

THE STRUCTURE OF THE MONOSACCHARIDE MOLECULE

THE CHEMICAL STRUCTURE OF GLUCOSE.—The chemical configuration of the glucose molecule has been derived from a series of organic studies.

1. By elementary analysis and molecular-weight determinations, the formula has been shown to be $C_6H_{12}O_6$.

2. Reduction with hydriodic acid yields *n*-secondary-hexyliodide, $CH_3—CH_2—CHI—CH_2—CH_2—CH_3$, which is a derivative of *n*-hexane. All aldohexoses contain the *n*-hexane chain. Therefore, the straight-chain formula for the carbon portion of the molecule is proved.

3. The glucose molecule contains 5 hydroxyl groups, inasmuch as it forms a pentaacetyl glucose when treated with acetyl chloride or acetyl anhydride.

4. Glucose contains an aldehyde or a potential aldehyde group, inasmuch as it adds hydrocyanic acid to form a cyanhydrin; it reduces alkaline copper solution; it forms oximes with hydroxylamine, and hydrazones (and osazones) with hydrazines.

5. On oxidation, glucose yields a monobasic acid (gluconic acid) containing 6 carbon atoms. Therefore, the carbonyl group is on one of the terminal carbon atoms. Fructose under the same conditions of oxidation breaks up, yielding, among other products, trihydroxybutyric acid.

6. The stability of the molecule is evidence that not more than one hydroxyl group is on any one carbon atom.

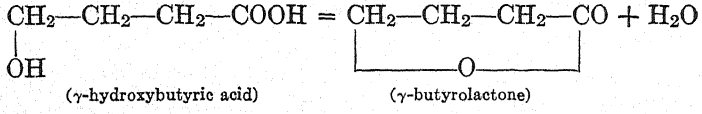
Since there are 5 hydroxyl groups and a carbonyl group on a terminal carbon atom this yields

$$CH_2OH—CHOH—CHOH—CHOH—CHOH—CHO$$

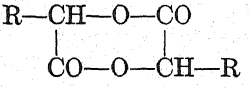
as the formula of glucose. This formula, however, does not give us any information about the space configuration of the asymmetric carbon atoms. These space configurations will be considered later. All the hexoses, with the exception of hamamelose,[1] have the same straight-chain carbon formula as noted above, when we consider the aldehyde group as being free.

[1] This unusual sugar, which occurs in the tannin of *Hamamelis virginica*, has the structure $CH_2OH \cdot CHOH \cdot CHOH \cdot C(OH)\begin{cases} CH_2OH \\ CHO \end{cases}$

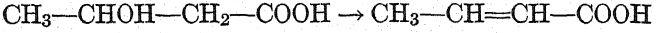
THE CLOSED-CHAIN FORMULA OF THE MONOSACCHARIDES.—Certain hydroxy compounds readily lose water or undergo rearrangements, with the formation of ring structures. Thus, the γ-hydroxy acids lose water to form γ-lactones or anhydrides, the ring containing 4 carbon atoms and 1 oxygen atom.



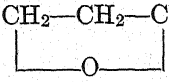
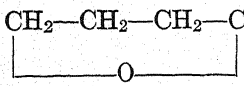
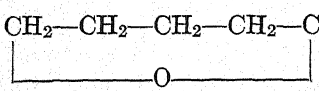
The name *lactone* is applied only to rings produced by internal anhydride formation from an hydroxy acid. Thus, two molecules of α-hydroxy acids usually combine to form an anhydride of the type



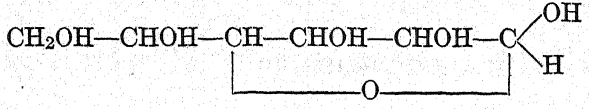
β-hydroxy acids lose water to form unsaturated compounds,



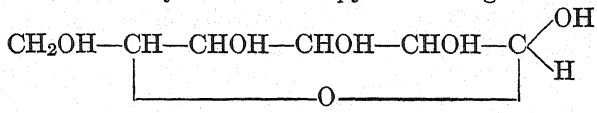
and γ and δ acids to form lactones.

When oxygen rings are formed from organic compounds other than acids, they are known as *oxides*. Thus, we may have an α-oxide or ethylene oxide ring with the formula,  , a β-oxide or propylene oxide ring,  , a γ-oxide or butylene oxide ring,  , or a δ-oxide or amylene oxide ring,  , etc.

Tollens, in 1883, proposed a closed-ring formula for glucose containing 4 carbon atoms and an oxygen atom. This formula was generally adopted, inasmuch as it appeared to represent the reactions of the carbohydrates more accurately than did the straight-chain formula. An oxide containing 4 carbon atoms would be a γ-oxide, and in the older literature the closed-ring formula of the aldohexoses is figured as a γ-oxide or butylene oxide ring.



Recent researches have indicated very clearly that in most instances the ring is not a γ-oxide or butylene oxide or furanose ring but rather a δ-oxide or amylene oxide or pyranose ring.



This point will be referred to again.

The straight-chain formula provides for only one modification of *d*-glucose, whereas the oxide formula allows for two sugars having the same space relations as *d*-glucose, owing to the fact that there is a possibility of isomerism in the terminal carbon atom of the oxide ring, thus introducing a new asymmetric carbon atom. *Two forms of d-glucose have actually been isolated.* These we know as α-*d*-glucose and β-*d*-glucose. Since the carbon atom is tetrahedral with the points of attachments to other groups at the apexes of the tetrahedron, a carbon-carbon linkage represents an angle of attachment between the carbon atoms of 109° 28′, so that even the straight-line formula represents a zigzag arrangement of the carbon atoms which coils back upon itself in the form of a spiral. Thus, even a straight-chain compound cannot be accurately depicted in a plane surface diagram, for it really has three dimensions. In depicting the ring structure of the sugars, Haworth introduced the perspective formula, noting that all carbon atoms were not in the same plane and that probably the carbon atoms (and the oxygen atom) occupy space relations somewhat similar to those shown in Fig. 142. Haworth then proceeded to depict the space relationships of the groups attached to the carbon atoms in the various stereoisomeric forms of the pyranose sugars by a hexagonal plane surface in which the groupings which are normally written on the right-hand side of the vertical line of the carbon chain are depicted as projecting below the plane of the hexagon, with the corresponding left-hand groupings projecting above the plane of the hexagon. Haworth's formulas for the α- and β-*d*-glucoses may be depicted as:

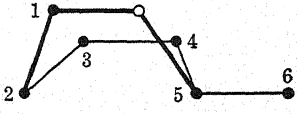
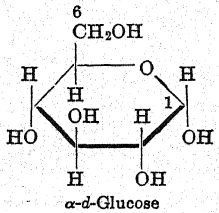
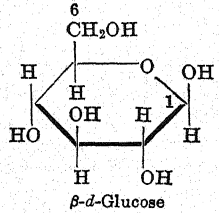


FIG. 142.—Space arrangement of the carbon atoms in the pyranose form of a monosaccharide. (After Haworth.)





In these formulas α-glucose has been written with the —OH group on carbon-1 situated on the same side of the carbon chain as is the —OH group on carbon-2. This is in agreement with the findings of Böeseken,[2] who, in the investigation of the behavior of polyhydroxybenzene derivatives and of glycols in solutions of boric acid, found that those compounds which possessed two —OH groups in the *ortho-* position or on the same side of the carbon chain combined with the boric acid and increased the electrical conductivity of the boric acid solution. When he came to investigate the sugars he found that α-glucose increased the electrical conductivity of boric acid solutions and that when mutarotation occurred the conductivity decreased. Accordingly he suggested that in α-glucose the hydroxyl groups on carbon-1 and carbon-2 were on the same side of the chain. In the subsequent formulas the author has adopted this convention which, in the case of glucose and galactose, appears to be the actual configuration. In the case of fructofuranose (γ-fructose) the α- and β-forms have been depicted as suggested by Isbell.[3]

[2] Böeseken, J., L'influence de quelques glycols sur la conductibilité électrique de l'acide borique, *Rec. trav. chim.*, 34: 96–113 (1915); Über die Lagerung der Hydroxyl-Gruppen von Polyoxy-Verbindungen im Raum. Die Konfiguration der gesättigten Glykole und der α- und β-Glykose, *Ber.*, 46: 2612–2628 (1913); Böeseken, J., and Couvert, H., La configuration de quelques sucres, *Rec. trav. chim.*, 40: 354–380 (1921).

[3] Isbell, H. S., personal communication. Dr. Isbell comments on these problems as follows: "The recent work of Hudson and Jackson confirms Böeseken's assignment of structure to α-methyl-*d*-glucopyranoside and the subsequent classification of the other methyl-aldopyranosides by means of the optical rotations. It is therefore possible to assign structures to the α- and β-aldopyranoses with reasonable certainty. Our knowledge of the ketose series is less secure, although it is generally assumed (and probably correctly) that the substitution of the hydrogen on carbon-1 of the aldose by CH_2OH does not cause a reversal in the sign of the optical rotation. This is not proven and assignment of structures from optical rotation in the ketose series must be considered provisional. Perhaps the oxidation method of Hudson and Jackson will furnish an experimental basis in the future. The fructofuranoses are not known substances at the present time and therefore their formulas represent hypothetical substances which we would call by that name. The glycosidic group in the α-*d*-pyranose is the mirror image of that in the α-*l*-pyranose so that the designation α (or β) does not represent the absolute configuration of the glycosidic group. In order that the two enantiomorphic substances may be designated in like manner, it is necessary to take into account the configuration of the glycosidic carbon and a second group. At a time when the ring structures were not known, Dr. Hudson selected the terminal asymmetric carbon as the key group. In many cases the terminal asymmetric carbon forms the oxygen ring so that its configuration determines the configuration of the ring; in other cases it lies in the side chain and does not materially affect the structure of the glycosidic carbon. The configurations of the atoms comprising the pyranose ring determine in large measure the properties of the sugars, and for this reason I suggested that the configuration of the oxygen ring (carbon-5 in the aldopyranoses) be used for naming the α- and β-sugars and glycosides (rather than the configuration of the terminal asymmetric carbon).

Isbell [4] also notes that Hudson [5] originally designated the more dextrorotatory sugar of an α-β pair as α in the *d*-series and β in the *l*-series but that this suggestion was made before we had any knowledge as to the position of the oxygen ring. Isbell then points out that in the normal aldoses the hydroxyl attached to carbon-5 forms the oxygen ring, so that the configuration of this hydroxyl determines the direction in which the ring is bent. In all the normal aldo-*d*-hexoses this hydroxyl lies to the right, and in all the normal aldo-*l*-hexoses it lies to the left. Therefore, the configuration of the hydroxyl group on carbon-5 determines the position of the oxygen ring.

Isbell further points out that when the glycosidic hydroxyl lies in the same direction as the ring oxygen, the sugar reacts with bromine water more slowly than when the glycosidic hydroxyl is directed away from the ring, and he proposes the following rule: "*When the oxygen ring lies to the right, as in d-glucose, the more dextrorotatory member of the α-β pair shall be designated α, and the less dextrorotatory member β; when the oxygen ring lies to the left, as in l-glucose, the more levorotatory member shall be designated α, and the less levorotatory β. This rule is applicable to the furanoses as well as the pyranoses and to the derivatives of both.*" He then goes on to point out that the same rule can be applied to the pentoses, since α-*d*-xylose, β-*d*-lyxose, β-*d*-ribose, and *l*-arabinose are genetically related to the *d*-hexoses, and their oxygen rings lie to the right. Therefore, the more dextrorotatory forms of these sugars are properly designated as the α-forms.

The space configuration of a three-dimensional model of β-*d*-glucose is shown in Fig. 143*A*. In Fig. 143*B* are shown space models of the open-chain formula and the pyranose formula of α-*d*-glucose.

Sponsler and Dore,[6] from an investigation of the X-ray crystal structure of α-*d*-glucose, conclude that it has a molecular volume of 193 Å^3. With four molecules of α-*d*-glucose in the crystal lattice, it thus occupies a parallelogram 10.45Å × 14.85 Å × 4.97 Å. The monohydrate has a molecular volume of 207.9 Å^3, two molecules being in the crystal lattice which has a space volume of 8.72 Å × 5.03 Å × 9.59 Å.

Application of this system requires changing the names of the pyranose derivatives of arabinose, fructose, and certain heptoses. Since the hydroxyl on the key carbon (carbon-5) in fructofuranose determines the *d*-classification and forms the furanose ring, there is no difference in this case in the nomenclature of Hudson and that proposed by me. In both systems the glycosidic hydroxyl lies to the right when the formula is written in the Fischer projectional manner."

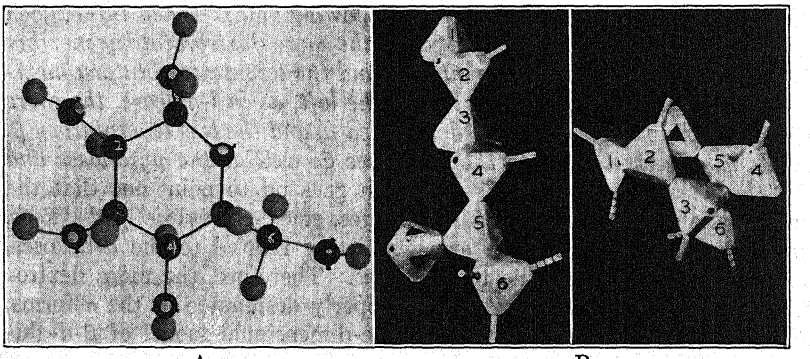
[4] Isbell, H. S., Nomenclature of the Alpha and Beta Sugars, *J. Chem. Education*, 12: 96 (1935).

[5] Hudson, C. S., The Significance of Certain Numerical Relations in the Sugar Group, *J. Am. Chem. Soc.*, 31: 66–86 (1909).

[6] Sponsler, O. L., and Dore, W. H., The Crystal Structure of Some Forms of Glucose. A Preliminary Paper, *J. Am. Chem. Soc.*, 53: 1639–1643 (1931).

The aldehyde reactions are explained by a rupture of the oxide ring and the formation by the addition of water, first of an aldehydrole, $-CH(OH)_2$, then by a loss of water from the aldehydrole to form an aldehyde group. The oxide ring structure thus allows for 5 asymmetric carbon atoms in place of the 4 of the straight-chain formula, and for 32 instead of 16 isomeric aldohexoses.

Since 1920 there has been a drastic revision of the structural formulas [7] of most of the simple sugars. Haworth and his school have been particularly active in these studies. The naturally occurring sugars, xylose, arabinose, rhamnose, glucose, fructose, galactose, and mannose, all possess the amylene oxide or pyranose structure, and in the case of the sugars which are linked in the di-, tri-, and polysac-



A B

FIG. 143.—(*A*) The space arrangement of a three-dimensional model representing the pyranose form of β-*d*-glucose. (After Sponsler and Dore.) (*B*) Space arrangement of the carbon atoms in the open chain and pyranose forms of α-*d*-glucose. (After Brauns.)

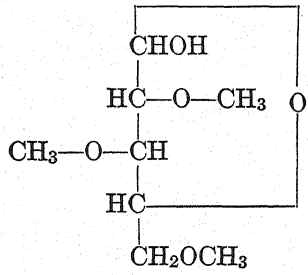
charides, the pyranose structure is very much more common than the γ-oxide or furanose structure.

Besides the α- and β-forms of sugars there exists a γ-form which cannot be isolated directly, because of its great reactivity. Derivatives, however, may be prepared, and such derivatives as tetramethyl-γ-*d*-glucose, trimethyl-γ-*d*-glucose, tetramethyl-γ-*d*-galactose, tetramethyl-γ-*d*-mannose, tetramethyl-γ-*d*-fructose, and trimethyl-γ-*d*-fructose have been prepared and isolated. *All undergo oxidation with great ease, e.g.*, trimethyl-γ-*d*-glucose reduces Fehling's solution in-

[7] For summary see Haworth, W. N., Ring Structure in the Mono-, Di-, and Poly-Saccharides, Rapports sur les hydrates de carbone, Tenth Conference of the International Union of Chemistry (Liége, September 14-20, 1930), Paris, 1931, pp. 33–58; also Haworth, W. N., The Constitution of Sugars, Longmans, Green and Company, New York (1929), and Armstrong, E. F., and Armstrong, K. F., The Carbohydrates, Fifth Edition, Longmans, Green and Company, Monographs on Biochemistry (1934).

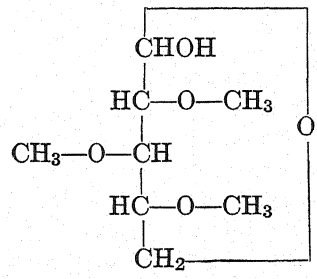
stantly at room temperature. At ordinary temperatures they form glycosides almost instantaneously, whereas it is necessary to heat the reaction mixture for 60 hours at 100° to form α-methylglucoside, where the sugar has the pyranose structure.

Apparently γ-glucose, which can be taken as a representative of the γ-sugars, differs from α- and β-glucose by not having the pyranose ring. Apparently all the γ-sugars have the furanose structure. This is true for the pentoses and for the hexoses. The contrast in the reactivity of the pyranose and furanose forms can be illustrated by the reactions of the trimethyl derivatives of the corresponding xyloses which possess the following structures with the corresponding physical and chemical properties:



Trimethyl-*d*-xylose (furanose)

Syrup, b.p. 110°/0.04 mm.
$[\alpha]_D = +24.7° \rightarrow 31.2°$
Reduces cold permanganate.
Combines with acid methyl alcohol.

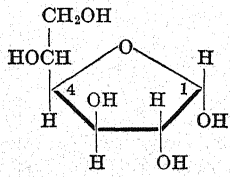


Trimethyl-*d*-xylose (pyranose)

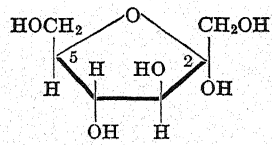
Crystalline, m.p. 87°–90°.
$[\alpha]_D = +74° \rightarrow 21°$.
No action with cold permanganate.
Slow combination with acid methyl alcohol.

It is impracticable to note the physical and chemical properties of the various derivatives which have been prepared, but the two inserted above may be regarded as typical. Tetramethyl γ-galactose readily undergoes spontaneous condensation with itself at room temperature to form a non-reducing disaccharide.

The furanose structures of *d*-glucose and *d*-fructose are indicated in the following diagrams:

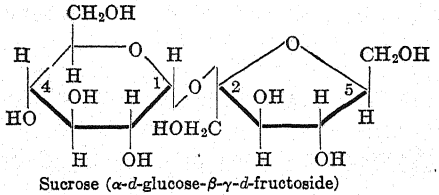


α-γ-*d*-Glucose



α-γ-*d*-Fructose

Sucrose may be regarded as having been formed through the condensation of one molecule of α-glucose, having the pyranose structure, with one molecule of β-fructose, having the furanose structure, with the elimination of one molecule of water. Sucrose, thus, has the formula:



Sucrose (α-*d*-glucose-β-γ-*d*-fructoside)

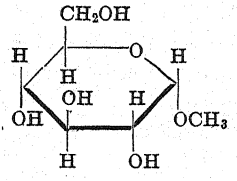
It is questionable whether the free aldehyde form of the sugars ever occurs in nature, although perhaps it may appear momentarily during some of the transformations which the sugars undergo. Wolfrom [8] prepared a new form of glucose pentaacetate which he regards as a derivative of the free aldehyde sugar. In this pentaacetate, the aldehyde group was not linked in an oxide ring but was free so that the pentaacetate was reducing, could form a cyanhydrin, and showed the other properties of free aldehyde groups.

Optieal Rotation and Ring Structure in the Sugars.—Hudson [9] and his co-workers have extensively investigated the relationships between molecular configuration and the optical rotatory power of the various sugars. Rules have been devised whereby the structure of a sugar can be predicted from a knowledge of the optical rotation, or *vice versa,* the degree of rotation can be predicted for a particular stereochemical configuration of a sugar. In the space at our disposal it is impossible to go into the details of these studies, but the student interested in the effect of organic structure on physicochemical behavior cannot afford to overlook these important contributions.

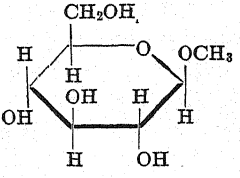
The Methylglucosides.—There are two isomeric methylglucosides derived from the α- and β-modifications of glucose. These are analogous to the naturally occurring glycosides. Fischer prepared these glucosides in 1893 by dissolving glucose in cold methyl alcohol and saturating the solution with dry hydrogen chloride. Their formulas may be represented as

[8] Wolfrom, M. L., The Acetate of the Free Aldehyde Form of Glucose, *J. Am. Chem. Soc.*, 51: 2188–2193 (1929).

[9] See numerous papers under the general title, "Relations between the Rotatory Power and Structure in the Sugar Group," in *J. Am. Chem. Soc.*, since 1924; *cf.* also Haworth, W. N., and Hirst, E. L., The Structure of Carbohydrates and their Optical Rotatory Power, I–V, *J. Chem. Soc.*, 2615–2663 (1930).



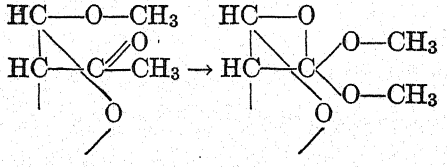
d-Glucose-α-methyl-glucoside



d-Glucose-β-methyl-glucoside

A study of these methylglucosides has given added assurance of the evidence of α- and β-forms of glucose in the ordinary glucose of the chemical laboratory.

In studying the methylglucosides of acetylated sugars, Haworth,[10] *et al.*, isolated three tetraacetylmethyl-*d*-rhamnosides and three tetraacetylmethyl-*d*-mannosides. All were found to have the pyranose structure. One is the ordinary α-methylglucoside; another, the ordinary β-methylglucoside; and the third, which the authors call a "γ-glucoside," is due to a rearrangement of the methyl group, where the methyl group migrates from the methoxy to the acetyl group on carbon-2. This rearrangement forms a very stable linkage. The rearranged acetyl group on carbon-2 is not saponified even with hot alkali. The rearrangement is shown in the following diagram:



The methylglucosides never behave as aldehydes. The aldehyde group is entirely masked. Their rotatory power is the same in a freshly prepared solution as in an older solution. This is not true of glucose (*vide infra*) where the α- and β-forms are in equilibrium in different amounts under different conditions of temperature, concentration, etc. The methylglucosides may in turn be hydrolyzed to glucose and methyl alcohol. However, the same enzyme will not hydrolyze both, *i.e.*, we have specific enzymes for hydrolyzing the α- and β-glucosides. α-Methylglucosides are hydrolyzed by the enzyme *maltase*, the enzyme that splits the disaccharide, maltose, and the

[10] Haworth, W. N., Hirst, E. L., and Miller, E. J., The Development of a Novel Form of Stereoisomerism in the Sugar Series. I. The Third Variety of Triacetyl Methylrhamnoside, *J. Chem. Soc.*, 2469–2479 (1929); Bott, H. G., Haworth, W. N., and Hirst, E. L., II. The Third Variety of Tetra-acetyl Methylmannoside, *ibid.*, 1395–1405 (1930).

β-methylglucosides are hydrolyzed by the enzyme *emulsin*, the enzyme that hydrolyzes the naturally occurring glycosides. These enzymes act best at about 37° C. (time=several hours) and are more active hydrolyzing agents than acids.

When solid anhydrous glucose (largely α-glucose) is used for the preparation of the methylglucosides, the equilibrium mixture contains 77 per cent of the α-methylglucoside and 23 per cent of the β-isomeride. To prepare the pure β-form, a biological method is used. Ordinary bakers' yeast contains maltase which will split the α-form, and zymase which will ferment the resulting glucose to CO_2 and ethyl alcohol. Consequently, if a mixture of α- and β-methylglucosides is incubated with yeast, the α-methylglucoside is split, and the glucose is fermented, leaving the β-methylglucoside unaffected because no emulsin is present.

MUTAROTATION OR MULTIROTATION.—A freshly prepared solution of an aldo or keto sugar changes its optical rotation upon standing; sometimes the rotation increases or it may decrease, depending upon the sugar being investigated. Eventually, however, a constant value is reached, the final reading depending upon the factors of concentration, temperature, and time.

With anhydrous glucose the rotation falls about 50 per cent. The hypothesis of two stereoisomeric forms of glucose is the only one that affords an adequate explanation. Lippmann,[11] in 1895, suggested that mutarotation in solution was due to a change from one oxide form to the other. At that time, only one anhydrous glucose was known, and the existence of isomers was purely hypothetical. In this and the following year (1895–1896), Tanret[12] isolated some new forms of glucose, galactose, lactose, arabinose, and rhamnose, and separated what he thought were three distinct forms of *d*-glucose.

1. α-Glucose with an optical rotation of + 110°, falling gradually to + 52.5°.

2. β-Glucose with an optical rotation of + 19°, rising gradually to + 52.5°.

3. "γ-Glucose" with an optical rotation of + 52.5° which remained constant in solution.

Simon[13] suggested that α- and β-glucose were homologs of α- and β-methylglucosides and that both contained a closed oxygen ring analogous to the glucosides whose structure had already been established.

[11] Lippmann, E. O. von, Die Chemie der Zuckerarten, Zweite Auflage, Braunschweig (1895).

[12] Tanret, C., Sur les modifications moléculaires du glucose, *Bull. soc. chim.*, (3) 13: 728–735 (1895); Sur les modifications moléculaires et la multirotation des sucres, *ibid.*, 15: 195–205, 349–361 (1896).

[13] Simon, L. J., Sur le constitution du glucose, *Compt, rend.*, 132: 487–490 (1901).

Armstrong [14] proved definitely that the α- and β-isomers have the oxide structure. He showed that enzymes hydrolyze the α- and β-methylglucosides to α- and β-glucose respectively, yielding sugars with (*a*) high or (*b*) low initial optical rotation, and that regardless of what the initial rotation is, the same equilibrium rotation is eventually reached. Armstrong gives the following values for optical rotation:

α-methylglucoside	$[\alpha]_D = +159°$	α-glucose	$[\alpha]_D = +113.4°$.
β-methylglucoside	$[\alpha]_D = -34°$	β-glucose	$[\alpha]_D = +19°$.
	α-β-equilibrium $[\alpha]_D = +52.2°$		

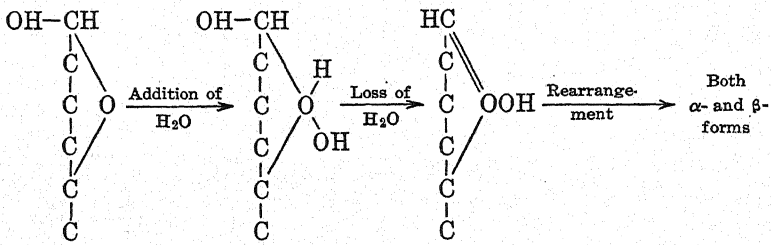
Tanret's "γ-glucose" proved to be the equilibrium mixture of the α- and β-modifications. Tanret, in 1905, accepted this view and calculated the equilibrium mixture of a 10 per cent glucose solution to contain 37 per cent of α-glucose and 63 per cent of β-glucose.

Glucose purified by crystallization from dilute methyl alcohol is invariably a mixture of α- and β-forms. Hudson and Dale [15] recommend acetic acid of different concentrations to separate, by fractional crystallization, the α- and β-modifications.

Two explanations have been advanced to account for the mechanism of the isomeric shift of $\alpha \rightleftharpoons \beta$.

1. Lowry considers the formation of the aldehyde or its hydrate to be an intermediate step, thus opening the oxide chain.

2. Armstrong believes that the oxide ring does not need to open. He considers that the first stage is the formation, by addition of water, of an *oxonium hydrate* which later loses water, a part of the elements of water coming from either the hydrogen or hydroxyl group attached to the terminal carbon atom, the intermediate compounds so formed rearranging to a mixture of the α- and β-forms.



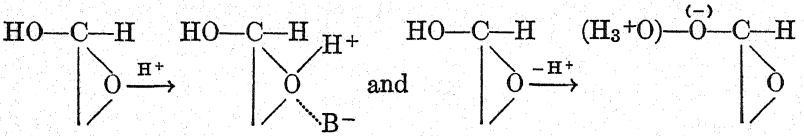
More recently [16] the evidence in favor of the intermediate forma-

[14] Armstrong, E. F., Studies on Enzyme Action. 1. The Correlation of the Stereoisomeric α- and β-Glucosides with the Corresponding Glucoses, *J. Chem. Soc.*, 83: 1305–1313 (1903).

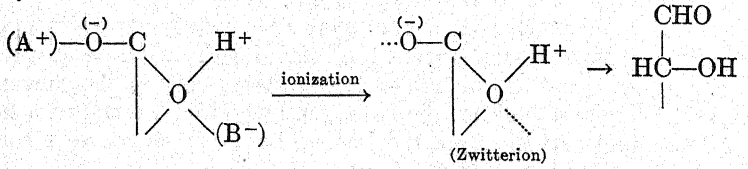
[15] Hudson, C. S., and Dale, J. K., Studies on the Forms of *d*-Glucose and Their Mutarotation, *J. Am. Chem. Soc.*, 39: 320–328 (1917).

[16] Lowry, T. M., Studies of Dynamic Isomerism. XVIII–XX, *J. Chem. Soc.*, 127: 1371–1401, 2883–2887 (1925).

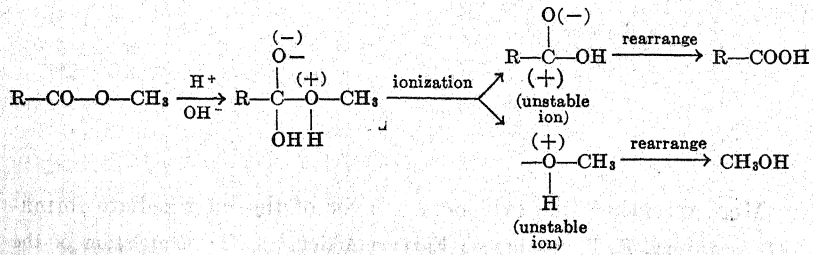
tion of an aldehyde has been strengthened. Lowry points out that the free aldehyde form (or an aldehydrol) must be an intermediate and that, in order for mutarotation to take place, the compound must receive a proton from a "proton donator" and yield a proton to a "proton acceptor." Water is both a proton donator and a proton acceptor, which explains mutarotation in aqueous solutions. He finds that, in pure dry *o*-cresol (a proton donator), there is no mutarotation, and in pure dry pyridine (a proton acceptor) there is no mutarotation, but in a mixture of 1 part pyridine to 2 parts *o*-cresol the mutarotation of sugars proceeds 20 times as fast as in pure water. Lowry pictures this as being due to the formation of a zwitterion:



Both reactions proceed simultaneously in an amphoteric solvent, or in a mixture of an acid and a base. We would have, under such conditions, the addition of both the acid and the base to the sugar molecule which on ionization would give rise to a zwitterion, and this by an electron transfer within the molecule would become the neutral aldehyde:



The above reactions are similar to the modern conception of the mechanism of the hydrolysis of esters considered as an ionic reaction:



The problem of mutarotation in the furanose sugars has recently been investigated by Isbell,[17] who finds that "the mutarotation of fructose consists in the change of the *α-d*-fructopyranose to a fructofuranose and that this reaction is analogous to the rapid reaction found

[17] Isbell, H. S., personal communication.

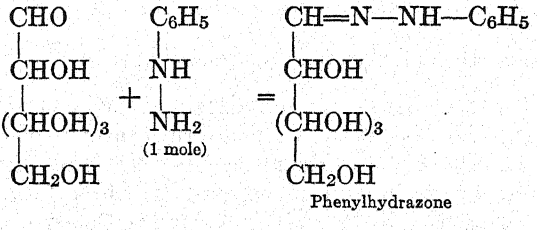
in the mutarotation of galactose. There is no evidence for the interconversion of the α- and β-pyranose modifications in the course of the mutarotation. This conclusion is supported by the catalytic effect of acids and bases, by the temperature coefficients for the reaction rates, by the heats of reaction, by volume change, and by other physical constants."

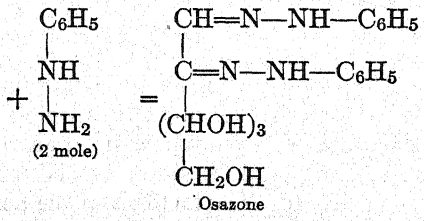
REACTIONS OF SUGARS WITH PHENYLHYDRAZINE.—Sugars react with phenylhydrazine in dilute acetic acid solution to form *phenylhydrazones* as the first product.

$$R{-}CHO + C_6H_5{-}NH{-}NH_2 = R{-}CH{=}N{-}NH{-}C_6H_5 + H_2O$$

The phenylhydrazone can be decomposed and the original sugar regenerated by acting on the phenylhydrazone with benzaldehyde, resulting in forming the phenylhydrazone of benzaldehyde at the expense of the sugar phenylhydrazone. Most of the phenylhydrazones of the sugars are easily soluble. Mannose is an exception, since it forms an almost insoluble phenylhydrazone, thus affording another striking illustration of the effect of molecular configuration on physical properties.

Excess of phenylhydrazine acts as an oxidizing agent toward the phenylhydrazone, converting the alcohol group on carbon-2 into a carbonyl group. This carbonyl group will then react with another molecule of phenylhydrazine to form an *osazone*. In the following formulas the hydrazone and the osazone are written as straight-chain compounds. It seems probable, however, that they likewise exist in the oxide-ring forms, since they undergo mutarotation in aqueous alcohol or aqueous pyridine solutions. Haworth figures glucosazone as having the pyranose structure.



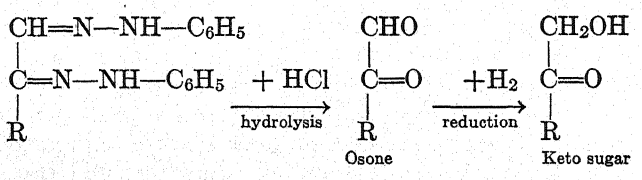


Glucose, mannose, and fructose yield the same osazone because the asymmetry of carbon-2 is destroyed in the formation of the osazone.

This indicates that the remaining four carbon atoms have the same molecular configuration in all three sugars. Glucosamine gives the same osazone as glucose, indicating that the $—NH_2$ group is on carbon-2. Because of the fact that the asymmetry of carbon-2 is destroyed, one should not place too much confidence in the properties of an osazone in identifying a sugar.

Butler and Cretcher [18] have defined special conditions under which glucose and fructose in the presence of one molecule of phenylhydrazine give rise to glucosazones. Mannose under the same conditions gives rise to the phenylhydrazone. When glucose- and fructose-hydrazones are allowed to stand in cold dilute acetic acid, a rearrangement takes place and a part of the hydrazones are changed to osazones. Mannose hydrazone under similar conditions is unaffected.

When an osazone is treated with fuming hydrochloric acid, both phenylhydrazine groups are split off and an *osone* results. Here, again, glucose, fructose, and mannose give the same osone. This is a colorless, strongly reducing syrup. On reducing this osone, *d*-fructose is obtained.



This is the only method available for regenerating a sugar from an osazone. The method is of historical interest, for Fischer established by its use the structure of synthetic α-acrose. It affords the means of preparing the corresponding *ketose* from an *aldose.*

Aldose → hydrazone → osazone → osone → ketose

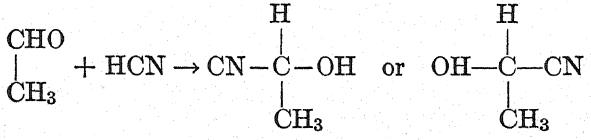
The asymmetric disubstituted hydrazines, such as methylphenylhydrazine, $C_6H_5—NCH_3—NH_2$, do not form osazones with glucose because they cannot act as oxidizing agents. Fructose, however, which already has a carbonyl group on carbon-2 is able to form a characteristic methylphenylosazone. Methylphenylhydrazine also forms a characteristic hydrazone with galactose, and diphenylhydrazine forms a characteristic hydrazone with arabinose.

THE CYANHYDRIN SYNTHESIS OF KILIANI.—Both aldehydes and ketones react with HCN to form nitriles which, when hydrolyzed, give acids containing one carbon atom more than the original aldehyde or ketone. Consequently, this reaction can be utilized to go from a C_5 sugar to a C_6 sugar, etc. The lactones of the acids formed from carbo-

[18] Butler, C. L., and Cretcher, L. H., New Conditions for the Formation of Glucosazone, *J. Am. Chem. Soc.*, 51: 3161–3165 (1929).

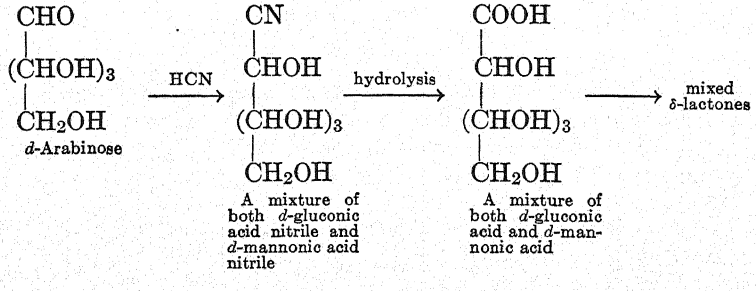
hydrates by this reaction can be reduced with sodium amalgam to yield the corresponding aldose containing one more carbon atom than the original aldose.

Two stereoisomeric nitriles are usually formed at the same time, for we are introducing a new asymmetric carbon atom into the aldehyde formula of the sugar,

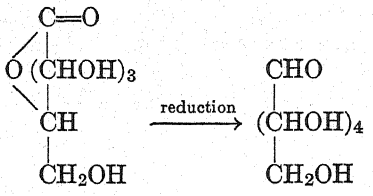


The synthesis should, and usually does, result in a racemic mixture of *d*- and *l*-forms; however, *mannose* and *fructose* are reported to form only a single nitrile. This would be an *asymmetric synthesis*, resulting in an optically active product. Such syntheses are very rare in the organic laboratory. We do not know the explanation for any one of the few asymmetric syntheses which are on record. If only a single nitrile were formed, it would probably mean that only the α- or the β-form of the sugar was present in the reaction mixture. In the case of mannose, Haworth [19] points out that mannose forms both α- and β-methylmannosides and not exclusively the α-form as the literature would indicate. Possibly the statement in the literature regarding the formation of a single nitrile is due to the failure to isolate the corresponding antimere.

By Kiliani's method we can advance from formaldehyde to a biose, and so on, one carbon at a time, to the higher sugars. Fischer carried glucose and mannose up to *aldononoses* (C_9). *d*-Arabinose yields two hexoses, *d*-glucose and *d*-mannose, indicating that the only difference in the structural configuration of glucose and mannose must be on carbon-2.



[19] Bott, H. G., Haworth, W. N., and Hirst, E. L., The Structure of Carbohydrates and their Optical Rotatory Power. Pt. IV. Derivatives of α- and β-Methylmannopyranoside *J. Chem. Soc.*, 2653–2659 (1930).



A mixture of the δ-lactones of *d*-gluconic and *d*-mannonic acid

A mixture of *d*-glucose and *d*-mannose

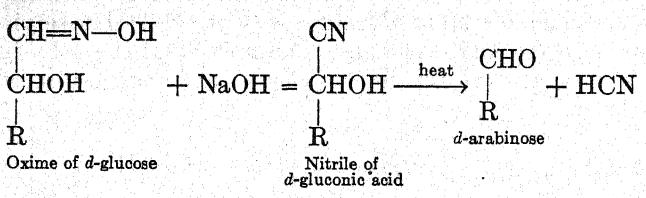
The acids may be separated by fractional crystallization before the *lactones* are reduced. An alternative view starts with the oxide form of the sugar (the more or less equilibrium mixture of α- and β-forms). The oxide ring breaks and adds HCN. The presence of α- and β-forms in unequal amounts explains why the corresponding nitriles are formed in unequal amounts. *d*-Arabinose yields a preponderance of the levorotatory mannonic acid.

THE DEGRADATION OF A SUGAR.—(Preparing carbohydrates which contain one less carbon atom than the original sugar.)

1. In *Wohl's method* the aldoxime is prepared from the aldose by condensation with hydroxylamine,

$$R—CHO + NH_2OH = R—CH=N—OH + H_2O$$

By treating the aldoxime of glucose, for example, with concentrated sodium hydroxide, it is converted into the nitrile of gluconic acid, and on heating this nitrile, HCN is eliminated and a pentose is formed.



Oxime of *d*-glucose

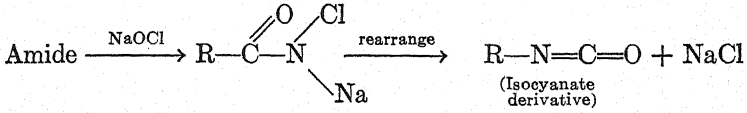
Nitrile of *d*-gluconic acid

d-arabinose

In practice it is preferable to heat the oxime with acetic anhydride and anhydrous zinc chloride. A vigorous reaction results, yielding the pentaacetyl derivative of gluconic acid nitrile, from which the —CN is eliminated by ammoniacal silver oxide.

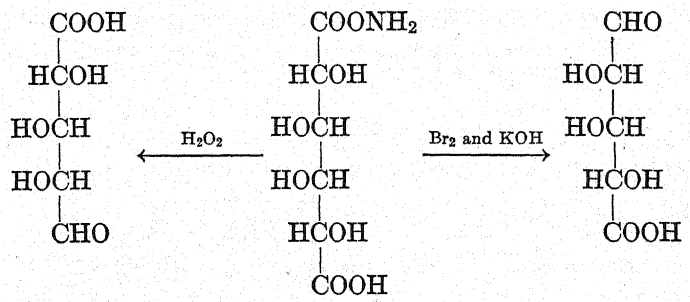
2. In *Ruff's method* the aldose is oxidized to the corresponding acid, and the calcium salt of the acid is further treated with hydrogen peroxide in the presence of ferrous ions, resulting in the loss of carbon dioxide from the acid and the formation of a pentose. Neuberg electrolyzes the copper salt of the acid, using platinum electrodes, and obtains reduction to the pentose and carbon dioxide.

3. *Weerman's method*[20] can be illustrated by the reactions of glucose. *d*-Glucose is oxidized to *d*-gluconic acid which spontaneously forms *d*-gluconolactone. The amide of *d*-gluconic acid is then prepared, and this is treated with sodium hypochlorite, resulting in the following sequence of reactions:



On treatment of the isocyanate derivative with sodium hydroxide, it breaks down, yielding *d*-arabinose and sodium isocyanate. Using this method Weerman secured a yield of 49.4 per cent of the theoretical *d*-arabinose from *d*-gluconamide, and 47.5 per cent of the theoretical *d*-lyxose from the amide of galactonic acid. More recently Haworth and Hirst used the method to prepare *l*-lyxose from *d*-galacturonic acid, the *l*-lyxose being used in their synthesis of *l*-ascorbic acid.

In the degradation of mucic acid Bergmann[21] somewhat modified Weerman's method, using the semi-amide of mucic acid. He found that hydrogen peroxide would oxidize the free carboxyl group, forming the amide of *l*-lyxuronic acid, from which the free amide could be obtained. Alkaline hypobromite solution would oxidize the amide group and yield a *d*-lyxuronic acid.



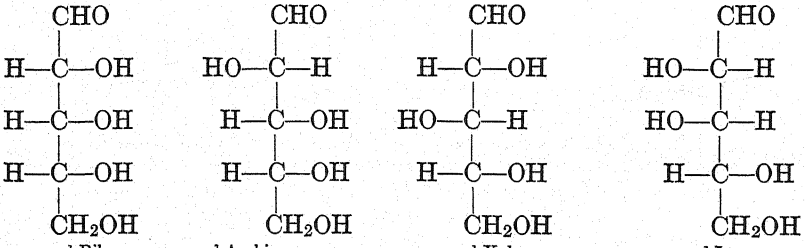
By one of the above methods, almost every sugar has been degraded, and in this way sugars have been secured which do not occur in nature and whose syntheses offer considerable difficulty.

[20] Weerman, R. A., L'action de l'hypochlorite de sodium sur les amides d'α-hydroxy-acides et de polyhydroxy-acides, ayant un groupe hydroxyle à la place α. Nouvelle méthode de dégradation des sucres, *Rec. trav. chim.*, 37: 16–51 (1918).

[21] Bergmann, M., Über den oxydativen Abbau von Schleimsäure und Zuckersäure zu neuen Aldehydsäuren der Zuckergruppe, *Ber.*, 54: 1362–1380 (1921); and Abbau von *d*-Zuckersäure zum Dialdehyd der *l*-Weinsäure, *ibid.*, 54: 2651–2658 (1921).

STEREOISOMERISM.—Determination of Configuration of Pentoses and Hexoses.[22]

I. *The Pentoses.*—There are eight possible aldopentoses, corresponding to the *d*- and *l*-forms of arabinose, xylose, ribose, and lyxose. There are thus four alternative forms for the *d*-modifications, which may be written as follows:



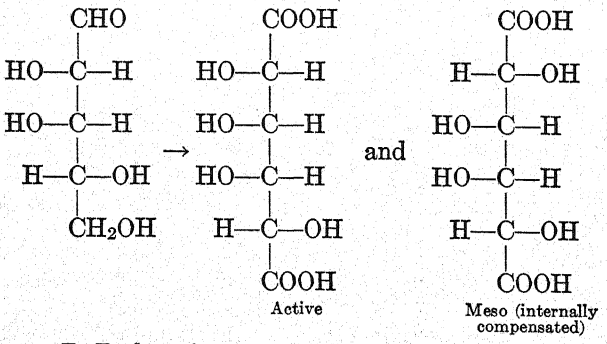
The *l*-forms are the mirror images of these.

A study of the various pentoses shows the following facts:

A. Arabinose and ribose form the same osazone. Therefore, their configuration must be identical except on carbon-2. Only (1) and (2) or (3) and (4) answer this arrangement.

B. Arabinose on oxidation gives an *optically active* dibasic acid, ribose and xylose yielding optically inactive compounds. Sugars (2) and (4) will give optically *active* dibasic acids: (1) and (3) will yield optically *inactive* dibasic acids. Therefore, arabinose is either (2) or (4), ribose and xylose are (1) and (3), and lyxose is either (4) or (2).

C. When HCN is added (Kiliani's reaction), a new asymmetric carbon atom is introduced into the molecule. The resulting hexose will therefore have two possible configurations. When the two hexoses from arabinose are oxidized, they give *optically active* acids, while



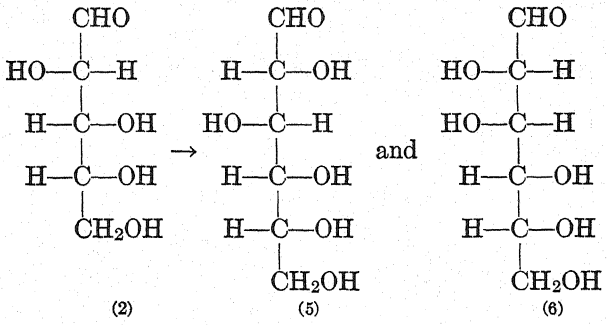
[22] Armstrong, E. F., *loc. cit.*; Stewart, A. W., Stereochemistry, Longmans, Green and Company, London (1907); the same methods which are used here are used to determine the structure of new compounds found in nature.

those from lyxose give two acids, one active and one inactive. Number (4) is the only one which will form both active and inactive C_6 dibasic acids.

Therefore, lyxose is (4), arabinose (2), ribose (1), and by elimination xylose is (3).

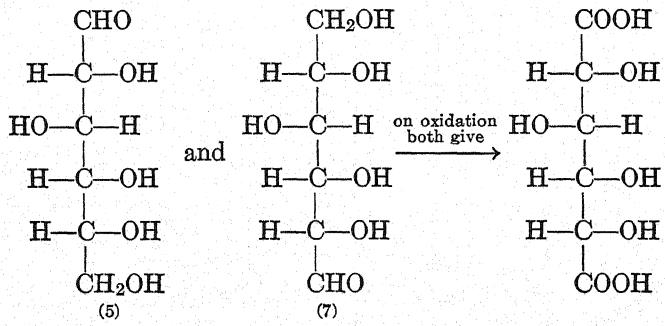
II. *The Hexoses.*—From the formulas of the pentoses we obtain the formulas of the hexoses. A study of the various hexoses shows the following facts:

A. Arabinose treated with HCN yields two hexoses, glucose, and mannose.



Therefore, glucose must be either (5) or (6).

B. Both glucose and gulose give the same dibasic acid on oxidation, *i.e.*, saccharic acid. This means the 4 asymmetric carbon atoms are the same. This can only occur when the terminal —CH_2OH and —CHO groups are interchanged as in (7).



In the case of (6) which is already symmetrical, it makes *no difference* in the transposition, *i.e.*, the same sugar results, no matter on which end, the —CHO and —CH_2OH groups occur. Therefore, glucose is (5), mannose is (6), and gulose is (7).

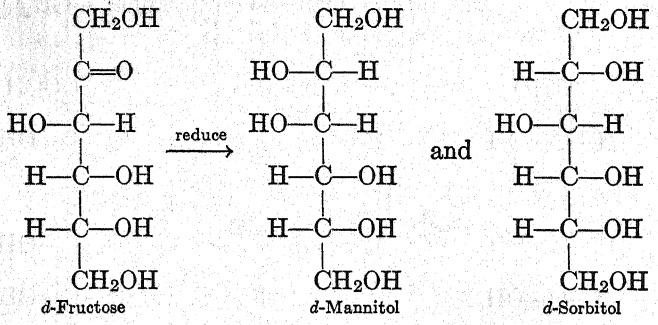
An extension of this reasoning gives the formulas of all the hexoses.

CHAPTER XXIV

CHEMICAL REACTIONS OF THE MONOSACCHARIDES

We have already discussed, in a consideration of the stereoisomerism of the pentoses and hexoses, the methods whereby monosaccharides are built up to sugars containing additional carbon atoms or whereby they are broken down to sugars containing a smaller number of carbon atoms. In this discussion it has been necessary to consider the reactions of the monosaccharides with phenylhydrazine, hydrocyanic acid, and hydroxylamine. In addition to these, a number of other reactions characteristic of the monosaccharides will be considered in this chapter.

THE REDUCTION OF THE MONOSACCHARIDES.—The reduction of a sugar produces a polyhydroxy alcohol. Only one alcohol is formed by the reduction of an aldose. Two isomeric alcohols are formed by the reduction of a keto sugar. It is by the reduction of a keto sugar that we are able to determine the configuration of keto sugars, such as fructose. Fructose gives rise upon reduction to two alcohols, *d*-mannitol and *d*-sorbitol.



d-Fructose *d*-Mannitol *d*-Sorbitol

The space formula for *d*-mannitol can be easily obtained from the corresponding formula of *d*-mannose. Similarly the space formula of *d*-sorbitol can be obtained from the space formula of *d*-glucose. The two alcohols differ only in the configuration of the groups attached to carbon-2. Therefore, the space configuration of the groups attached to the remainder of the fructose molecule is proved. The same line of reasoning has been applied to the determination of the structure of the C_7 sugar, sedoheptose.

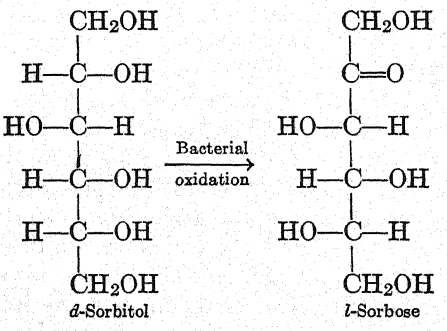
Both mannitol and sorbitol occur in plants. *d*-Mannitol is by far the most abundant of all the naturally occurring hexahydroxy alcohols. It occurs in the onion, carrot, turnip, pineapple, and in the higher and lower fungi. In the fungi especially it may exceed glucose in amount or even displace it entirely. It is a normal constituent of silage, being formed by the bacterial reduction of fructose (from sucrose).

Mannitol is readily soluble in hot alcohol, sparingly soluble in cold alcohol, which fact allows for its ready isolation by direct crystallization. The amount present in silage [1] ranges from 0.52 per cent to 2.54 per cent in average samples, although corn silage has been reported to contain as much as 3.3 per cent, and sunflower silage 5.61 per cent.

Toward the close of the World War the suggestion was made that nitromannitol be used as a high explosive, securing the mannitol from silage juices. *d*-Mannitol can easily be made by the catalytic hydrogenation of mannose.

d-Sorbitol occurs in the ripe fruits of the mountain ash and in most of the fruits of the *Rosaceae*. It can be readily prepared by hydrogenating *d*-glucose.

d-Sorbitol has taken on added interest within the last few years, inasmuch as ascorbic acid (vitamin C) is an oxidation product of *l*-sorbose which arises from *d*-sorbitol by the oxidation of the alcohol by means of various members of the *Acetobacter* group. Recently [2] conditions have been laid down under which a yield of 80–85 per cent of *l*-sorbose can be obtained from sorbitol by oxidizing the sorbitol with *Acetobacter suboxydans*.



d-Sorbitol *l*-Sorbose

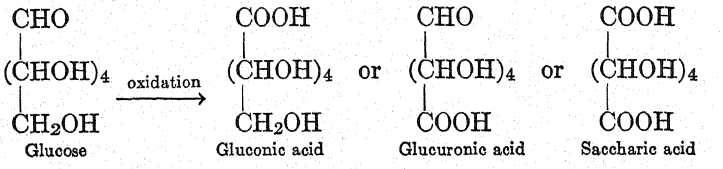
[1] Dox, A. W., and Plaisance, G. P., The Occurrence of Mannite in Silage and Its Possible Utilization in the Manufacture of Explosives, *Science*, 46: 192–193 (1917); The Occurrence and Significance of Mannitol in Silage, *J. Am. Chem. Soc.*, 39: 2078–2087 (1917); also *Research Bull.* 42, Iowa Agr. Exp. Sta., Dec., 1917.

[2] Fulmer, E. I., Dunning, J. W., Guymon, J. F., and Underkofler, L. A., The Effect of the Concentration of Sorbitol upon the Production of Sorbose by the Action of Acetobacter Suboxydans, *J. Am. Chem. Soc.*, 58: 1012–1013 (1936).

Perhaps a word of explanation should be inserted as to why an alcohol of a *d*-series gives rise upon oxidation to a keto sugar of the *l*-series. *d*-Fructose when reduced gives rise to *d*-sorbitol, but the oxidation of *d*-sorbitol does not involve carbon-2 which has been reduced; instead the oxidation with *Acetobacter* takes place on what was carbon-5 of fructose. Accordingly, when the formula of sorbose is written with the carbonyl group on carbon-2, the sorbose shows stereochemical relationships to *l*-glucose rather than *d*-glucose.

The alcohol of galactose, dulcitol, is particularly abundant in the red algae. It is not widely distributed in the higher plants.

THE OXIDATION OF THE SUGARS.—Glucose on oxidation yields three different acids, all containing six carbon atoms:



Glucose — Gluconic acid — Glucuronic acid — Saccharic acid

d-Glucose, when oxidized with bromine, yields *d*-gluconic acid. This acid is of interest, because, when it is heated with pyridine or quinoline, it is partly converted into its stereoisomeric isomer, or antimere, *i.e.*, *d*-mannonic acid. The gluconic to mannonic acid reaction is important, because it affords a means of passing from glucose to mannose, and in a similar way one may pass from galactose to talose, and from xylose to lyxose. Apparently the —H and —OH groups on the carbon atoms adjacent to the carboxyl group interchange positions. Pyridine and quinoline prevent the formation of the lactone of the acids which, if present, would prevent the change from taking place.

Spoehr has reported the presence of glucuronic acid as a plant constituent, and we have already seen that it is a constituent of chondrosin in the glycoproteins. Physiologically it is one of the most important of the oxidation products. It is apparently formed only when the aldehyde group is "protected" by being combined with some other compound in a glycoside-like linkage. It is frequently found in urine associated, in such a glycoside-like compound, with other substances. When certain substances, *e.g.*, *camphor*, are taken into the body and it is difficult for the body to oxidize them, they are in many instances combined in the body with glucose to form glucosides. One end of the glucose chain is thus protected from oxidation, but the other end oxidizes to a carboxyl group, so that a glucuronic acid compound results. These conjugated compounds are then excreted. This appears to be the way in which more or less injurious and difficultly oxidizable compounds are dealt with by both plants and animals, *e.g.*, the glycosides in plants which contain hydrocyanic acid, benzaldehyde, etc. It is ap-

parently a means which the organism uses to protect itself against toxic agents. The plant glycosides may be compared in physiological importance to the animal glucuronic acid derivatives.

Griffith [3] notes that, when sodium benzoate is administered to rabbits, a part of the benzoic acid is eliminated in combination with glycine, as hippuric acid, but that in spite of this fact a very appreciable amount is likewise excreted as benzoylglucuronic acid, which in certain of his experiments amounted to one-third of the total combined benzoic acid which was excreted.

Quick [4] observed that, when benzoic acid is fed to a dog, it is excreted not as hippuric acid but as β-*d*-glucuronic-acid-α-monobenzoate. Quick further notes that the linkage between the glucuronic acid and the benzoic acid is not a glycosidal linkage, for the aldehyde group is still free.

d-Galacturonic acid probably does not occur in the free form in nature but is widely distributed as one of the major constituents of pectin and many plant gums and mucilages, *e.g.*, gum acacia. It has also been reported to be present in hemicelluloses. On decarboxylation by heating with dilute mineral acids, it loses carbon dioxide and passes to *l*-arabinose. It is thought that *l*-arabinose arises in nature through the decarboxylation of *d*-galacturonic acid.

d-Mannuronic acid had not been reported in nature prior to its isolation [5] from the alginic acid of *Laminaria saccharina* and *Fucus serratus*.

Saccharic acid is formed by the action of nitric acid on glucose. The sparingly soluble monopotassium salt serves as a test for glucose. *Mucic acid* is the corresponding acid of galactose. Thirty-five to 40 per cent nitric acid at 85° will produce the best yields of mucic acid. It has a sandy, crystalline appearance and can be readily prepared commercially from the galactans of the wood of the western larch.

The *keto sugars* on oxidation break at the carbonyl group and give rise to two acids; fructose, for example, gives glycolic acid, $CH_2OH—COOH$, and trihydroxybutyric acid.

Jackson and Hudson [6] report a new and unusual type of oxidation in the sugar series. They were interested in studying the oxidation

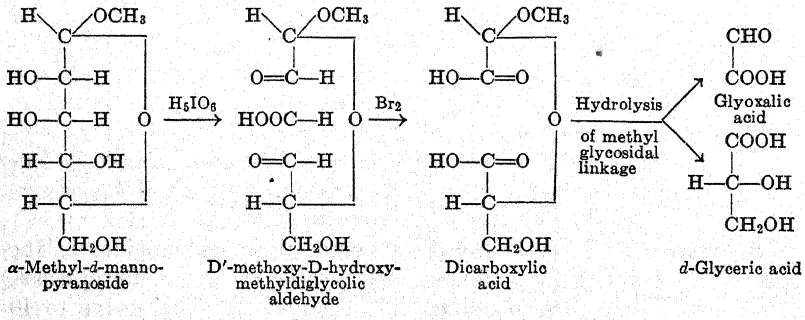
[3] Griffith, W. H., Benzoylated Amino Acids in the Animal Organism. III. A Method for the Determination of Hippuric Acid and a Study of the Synthesis of Hippuric Acid in Rabbits, *J. Biol. Chem.*, 69: 197–208 (1926).

[4] Quick, A. J., The Preparation and Study of β-*d*-Glycuronic Acid Monobenzoate (Benzoyl Glycuronic Acid), *J. Biol. Chem.*, 69: 549–563 (1926).

[5] Nelson, W. L., and Cretcher, L. H., The Properties of *d*-Mannuronic Acid Lactone, *J. Am. Chem. Soc.*, 54: 3409–3412 (1932).

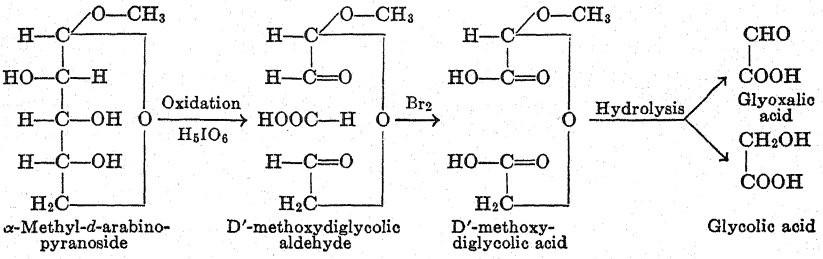
[6] Jackson, E. L., and Hudson, C. S., Studies on the Cleavage of the Carbon Chain of Glycosides by Oxidation. A New Method for Determining Ring Structures and Alpha Beta Configurations of Glycosides. *J. Am. Chem. Soc.*, 59: 994–1003 (1937).

products of the glycosides and anticipated that carbon-6 would be converted to a carboxyl group, thus giving rise to glycosides of the corresponding uronic acid. When α-methyl-*d*-mannopyranoside was oxidized with barium hypobromite, they found that carbon-3 was removed as formic acid, and the alcohol groups on both carbon-2 and carbon-4 were oxidized to a dibasic acid which they were able to isolate in excellent yield as the strontium salt. In studying the mechanism of the reaction they found that, if the mannoside was oxidized by periodic acid, carbon-3 was removed as formic acid, but carbon-2 and carbon-4 were oxidized only to the dialdehyde stage. This dialdehyde could then be further oxidized with bromine in the presence of strontium carbonate to yield the dicarboxylic acid which on hydrolysis of the methylglycosidal linkage breaks at what was the sugar-oxide ring to yield one molecule of glycolic acid and one molecule of *d*-glyceric acid.

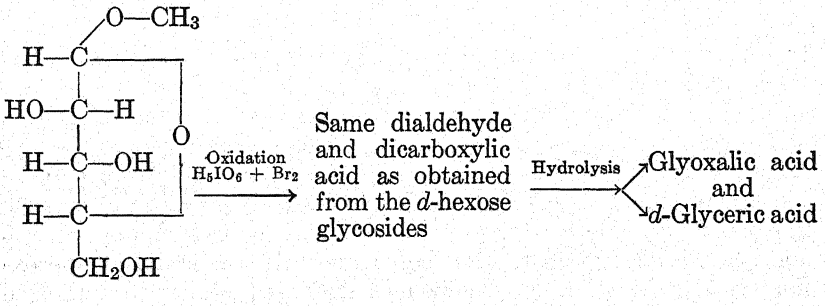


It will be noted that the oxidation even at the dialdehyde stage has destroyed all the asymmetric carbon atoms with the exception of carbon-5. Since carbon-5 is the carbon atom which determines the *d*-sugar series, it accordingly becomes apparent that *all the α-glycosides of the pyranose form of the d-sugars will give rise to the same dialdehyde and the same dicarboxylic acid.* Jackson and Hudson report that this was shown to be true experimentally in the case of *d*-mannose, *d*-galactose, *d*-glucose, and *d*-gulose. Furthermore, the formation of this dicarboxylic acid is added proof of the pyranose structure of the glycosides.

When Jackson and Hudson turned to the corresponding methylpyranosides of the pentoses, they found that the same mechanism held, that carbon-3 was eliminated, and a dicarboxylic acid was formed according to the following reactions. The dicarboxylic acid, when the glycoside was hydrolyzed, broke down to yield glyoxalic acid and glycolic acid:

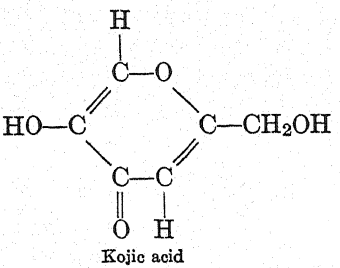


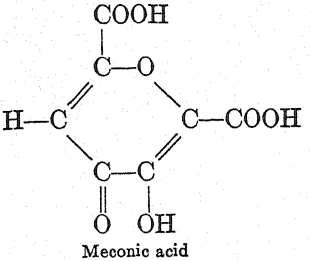
When, however, the third form of α-methyl-*d*-arabinoside was similarly oxidized, *the same dialdehyde and dicarboxylic acid were obtained as had been obtained from the d-hexose glycosides.* The diacid broke down to yield the same decomposition products as were yielded by the hexose glycosides, *i.e.*, glyoxalic acid and *d*-glyceric acid. This is interpreted to mean that the ring breaks between carbon-2 and carbon-3 of the furanose structure without the elimination of formic acid.

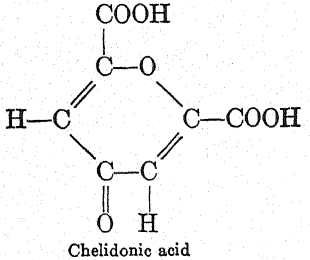


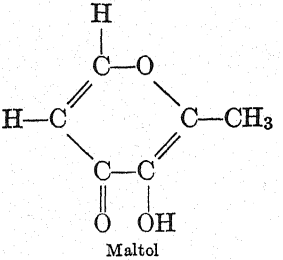
In the case of the pentose pyranosides this series of oxidations destroys all the asymmetric carbon atoms of the original pentose sugar. In the case of the pentose furanosides the asymmetry of carbon-5 is retained so that the *d*-pentoses yield *d*-glyceric acid, and *l*-pentoses would yield *l*-glyceric acid as final oxidation products. Hudson points out that this method of oxidation affords probably the easiest laboratory method of preparing *d*-glyceric acid. It also affords an independent method of determining structural affinities in the *d*- and *l*-sugar series and of determining whether a particular compound possesses the pyranose or the furanose structure.

A rather unusual oxidation product of the sugars is kojic acid, which was first isolated from the mycelium of *Aspergillus oryzae* which had been cultured on steamed rice.


Kojic acid


Meconic acid


Chelidonic acid


Maltol

(Formulas written to indicate relationships to fructopyranose)

It can be produced [7] in rather large quantities by growing *Aspergillus flavus* on a 20 per cent glucose solution. Under proper temperature and time conditions, 45 per cent of the dextrose in the culture solution had been transformed into kojic acid. While kojic acid has the pyranose structure, the ring is exceedingly stable, probably owing to the insertion of the double bonds. Physiologically it is quite toxic. When injected intravenously into dogs [8] it produced toxic symptoms at 150 mg. per kilo body weight, and the sodium salt was lethal at approximately 1 gram per kilo. Compounds somewhat similar to kojic acid occur in certain plants. Thus, chelidonic acid occurs in the leaves of the lily of the valley, meconic acid occurs with the opium alkaloids, and maltol occurs in pine needles and the bark of the larches.

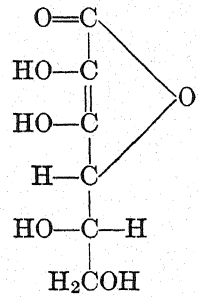
Ascorbic acid was discovered by Szent-Györgyi [9] when he was investigating the mechanism of biological oxidation and the function of the adrenal gland. He isolated a white crystalline compound which analyzed for $C_6H_8O_6$. It reduced silver salts in the cold, even in acid solution. He called this "hexuronic acid" and noted that it is the most reactive derivative of the carbohydrates hitherto discovered, which was obtainable in crystalline form. He found the same sub-

[7] May, O. E., Moyer, A. J., Wells, P. A., and Herrick, H. T., The Production of Kojic Acid by Aspergillus Flavus, *J. Am. Chem. Soc.*, 53: 774–782 (1931).

[8] Friedemann, T. E., Chemical and Physiological Properties of Kojic Acid, *Science*, 80: 34–35 (1934).

[9] Szent-Györgyi, A., Observations on the Function of Peroxidase Systems and the Chemistry of the Adrenal Cortex. Description of a New Carbohydrate Derivative, *Biochem. J.*, 22: 1387–1409 (1928).

stance to occur in the cortex of the adrenal glands and noted that it completely inhibited the formation of melanin pigments in all systems where the pigment is produced by the oxidation of a phenol. In 1930, he [10] suggested that its function in the adrenal gland may be to stabilize epinephrine and the other hormones of the cortex, and prevent their alteration by oxidation. Shortly after this, King and Waugh,[11] while attempting to isolate vitamin C, came to the conclusion that the product which they isolated and which they identified as vitamin C was identical with Szent-Györgyi's hexuronic acid. Ascorbic acid has been shown [12] to be a lactone and is the enol form of 3-keto-*l*-gulonolactone. It has been synthesized by methods starting with *l*-xylosone [13] and from glucose [14] which is reduced to sorbitol. This was oxidized to *l*-sorbose, and the *l*-sorbose diacetone derivative on oxidation yielded the diacetone derivative of 2-keto-*l*-gulonic acid. The free acid could be enolized by heating with water or with water saturated with carbon dioxide to yield ascorbic acid. Better results were obtained by enolizing with very dilute hydrochloric acid. It will be noted that the 2-keto acid and the 3-keto acid yield the same dienol derivative. Synthetic ascorbic acid is available commercially at a very moderate cost.



Ascorbic acid
(Vitamin C)
Enol form of
3-keto-*l*-gulonolactone)

THE ESTERS AND ETHERS OF THE MONOSACCHARIDES.—Since the sugars contain both the primary and secondary alcohol groups, they will form esters with acids, or ethers with methyl sulfate or methyl iodide in the same way that other primary and secondary alcohols form esters and ethers. Certain of these derivatives have proved of especial value in determining the configuration of the sugars. In the following paragraphs we will limit our discussion to glucose, but it should be understood the reactions indicated are typical of all the sugars.

The Sugar Ethers.—The five alcohol groups of glucose can be converted into the corresponding methyl ethers either by the method orig-

[10] Szent-Györgyi, A., On the Mechanism of Biological Oxidation and the Function of the Suprarenal Gland, *Science*, 72: 125–126 (1930).

[11] King, C. G., and Waugh, W. A., The Chemical Nature of Vitamin C., *Science*, 75: 357–358 (1931); Isolation and Identification of Vitamin C, *J. Biol. Chem.*, 97: 325–331 (1932).

[12] Herbert, R. W., Hirst, E. L., Percival, E. G. V., Reynolds, R. J. W., and Smith, F., The Constitution of Ascorbic Acid, *J. Chem. Soc.*, 1270–1290 (1933).

[13] Ault, R. G., Baird, D. K., Carrington, H. C., Haworth, W. N., Herbert, R., Hirst, E. L., Percival, E. G. V., Smith, F., and Stacey, M., Synthesis of *d*- and of *l*-Ascorbic Acid and of Analogous Substances, *J. Chem. Soc.*, 1419–1423 (1933).

[14] Reichstein, T., and Grüssner, A., Eine ergiebige Synthese der *l*-Ascorbinsäure (C-Vitamin), *Helv. Chim. Acta*, 17: 311–328 (1934).

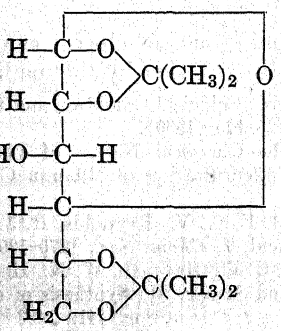
inated by Purdie and Irvine where the methyl glucoside is exhaustively treated with methyl iodide and silver oxide, or by the later method of Haworth, using methyl sulfate and alkali. When the glucosidal linkage is hydrolyzed, tetramethylglucose results. From glucopyranose this is 2-3-4-6-tetramethylglucose, from glucofuranose this is 2-3-5-6-tetramethylglucose, and it was by the use of the methyl derivatives that the space configuration of glucose was finally determined.

The great value of methylation is that the methyl ethers do not undergo stereochemical changes, such as racemization, Walden inversion, wandering of constituents, and changes in ring structure. Such changes do take place in the free sugars or in certain of the other derivatives. Therefore, by completely methylating an unknown sugar, the ring is fixed, and by identifying the particular carbons to which the methoxy groups are attached, one can determine the position of the oxide ring.

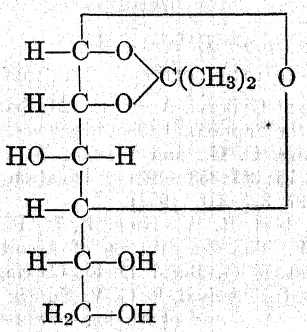
2-3-4-Trimethylglucose is obtained by hydrolyzing completely methylated gentiobiose, melibiose, and raffinose, as well as β-glucosan. This indicates the linkages of the glucose in these compounds.

2-3-6-Trimethylglucose arises as the hydrolysis product of completely methylated cellobiose, maltose, and lactose. It also arises as the hydrolysis product of trimethyl cellulose and trimethyl starch. Therefore, carbon-4 and carbon-5 are involved in the linkage to adjoining sugar residues and in the formation of the oxide ring.

Acetone Derivatives.—When a finely powdered sugar is suspended in acetone in the presence of a condensing catalyst, such as hydrochloric acid or zinc chloride, the acetone derivative is formed. Glucose yields a diacetone derivative which contains only one free hydroxyl group. This free hydroxyl group is on carbon-3. By controlled hydrolysis, glucose diacetone can be converted into glucose monoacetone in which carbon-3, carbon-5, and carbon-6 carry free hydroxyl groups. Glucose monoacetone and glucose diacetone both possess the furanose structure.

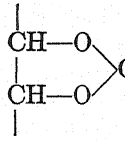


Glucose diacetone



Glucose monoacetone

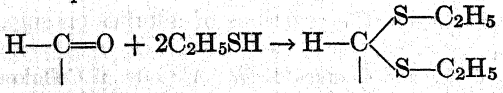
The acetone derivatives are relatively stable in the presence of alkalies but are readily hydrolyzed by dilute acids.

Sugar Carbonates.—By the use of carbonyl chloride, Haworth and Porter [15] prepared crystalline dicarbonate of the sugars containing the grouping  . In many respects these are analogous to the diacetone derivatives. They are somewhat more stable toward dilute acids, however.

Acyl Derivatives.—The hydroxyl groups of the sugars behave as alcohols. They can accordingly be esterified with acids, and the resulting acetates, benzoates, etc., have been widely used in the study of the structure of the carbohydrates, as well as in the preparation of various carbohydrate derivatives. The compounds containing the higher fatty acids, such as palmitic and stearic, likewise have been prepared and show characteristic properties. From the standpoint of structural chemistry, the acyl derivatives possess the disadvantage that the acyl group tends to migrate in later chemical reactions. Apparently migration tends to occur from a tertiary to a secondary alcohol group and from a secondary to a primary alcohol group.

Phosphoric acid esters are of importance in fermentation. The first stage in the fermentation of glucose by zymase appears to be the formation of hexose diphosphate in which phosphoric acid is esterified on carbon-1 and carbon-6. Glucose, mannose, and fructose give rise to the same hexose diphosphate, which probably is the explanation for the same ease of fermentation which is shown by these three sugars. When this diphosphate is hydrolyzed, fructose results. Two monophosphates have been prepared; in both instances the phosphoric acid appears to be esterified on carbon-6. One of these (the Robison ester) appears to be glucopyranose-6-phosphoric ester; the other (Neuberg's ester) appears to be fructofuranose-6-phosphoric ester. The former yields glucose on hydrolysis, the latter fructose. A synthetic glucose-3-monophosphoric ester can be prepared by the action of phosphorus oxychloride on glucose diacetone.

Sugar Mercaptals.—When sugars are treated in strong hydrochloric acid with mercaptans, the mercaptan condenses with the carbonyl group to form mercaptals:



[15] Haworth, W. N., and Porter, C. R., Sugar Carbonates. IV. The Dicarbonates of Glucose, Fructose, Mannose, Galactose, and Arabinose, *J. Chem. Soc.*, 151–157 (1930).

The condensation with the formation of the mercaptals is exceedingly rapid and takes place in a very few minutes even at 0°. The mercaptals are insoluble in water, easily crystallized from organic solvents, and may serve as an excellent means of characterizing specific sugars. Wolfrom and Georges [16] used mercaptalation to follow the hydrolysis of cellulose, noting that the hydrolytic products were converted into the mercaptals when the cellulose was dissolved in fuming hydrochloric acid and the solution kept at 0° in the presence of ethyl mercaptan. The ethyl mercaptal of glucose first appeared when hydrolysis of the cellulose was approximately two-thirds completed. When hydrolysis was approximately one-half completed, the mercaptal product isolated corresponded to the mercaptal of a trisaccharide. It seems probable that studies of this sort can be used to great advantage in studying linkages in the polysaccharides, since the mercaptals are nearly unique as reaction products in the rapidity with which the condensation takes place, in the low temperatures required for condensation, and in the resistance of the reaction product to subsequent hydrolysis.

Dissociation and Molecular Rearrangements. *The Action of Alkalies on Monosaccharides.*—The chemical rearrangements of the sugars have thrown light on reactions and physiological processes in metabolism. *In vitro,* pure solutions of sugars are stable. In the body they are extremely unstable and capable of numerous rearrangements and disintegrations. A study of the reactions of sugars to acids, bases, and salts may afford a clue to the reason for their behavior *in vivo,* including the mechanism of their decomposition and the nature of the products formed.

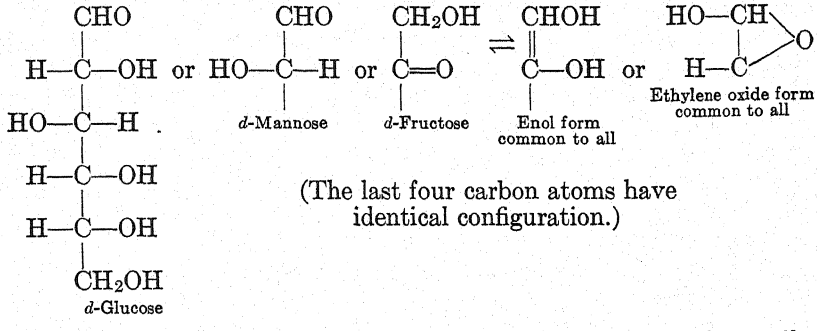
Sugars act as very weak acids. Michaelis and Rona [17] give the following dissociation constants at 18°: glucose, 6.6×10^{-13}; fructose, 9.0×10^{-13}; galactose, 5.2×10^{-13}; mannose, 10.9×10^{-13}; sucrose, 2.4×10^{-12}; maltose, 18.0×10^{-13}; lactose, 6.10×10^{-13}. That the sugars are *very* weak acids may be seen by comparison with the dissociation constants of some of the weak acids: acetic acid, 1.8×10^{-5}; butyric acid, 1.5×10^{-5}; lactic acid, 1.4×10^{-4}; boric acid (H^+ and H_2BO_4), 1.7×10^{-9}; and hydrocyanic acid, 4.7×10^{-10}. Ethyl alcohol forms sodium ethylate very easily; glycerol forms glycerolates with three sodium atoms. Sugars fall in the same class. Their importance lies in the fact that these metallic compounds decompose much more easily than the original sugar. Nef, over a period of more than ten years, studied the reactions of alkalies on sugars. He found

[16] Wolfrom, M. L., and Georges, L. W., A Study of Cellulose Hydrolysis by Means of Ethyl Mercaptan, *J. Am. Chem. Soc.,* 59: 282–286 (1937).

[17] Michaelis, L., and Rona, P., Die Dissoziationskonstanten einiger sehr schwacher Säuren, insbesondere der Kohlenhydrate, gemessen auf elektrometrischem Wege, *Biochem. Z.,* 49: 232–248 (1913).

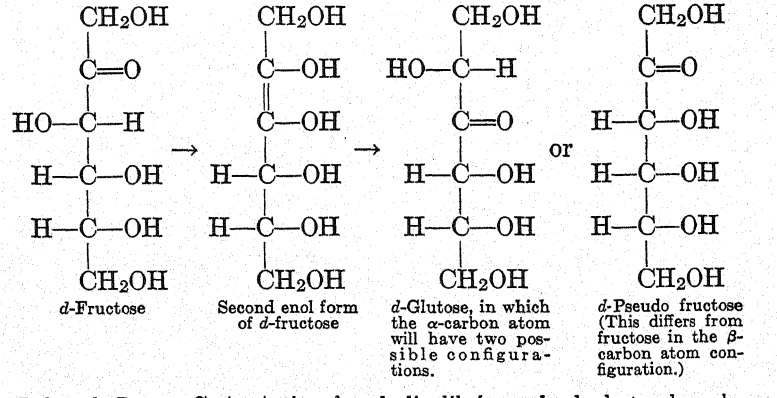
that all the compounds which were formed were very unstable and that they decomposed into a large number of substances.

A. If the concentration of hydroxyl ions is very low, a molecular rearrangement of the sugar molecule results. This rearrangement is known as the Lobry de Bruyn [18] transformation. Thus, if *d*-glucose is treated with a solution of 0.05 *N* calcium hydroxide, the optical rotation changes with time at 15° to 20° to a new equilibrium. Starting with either *d*-glucose, *d*-fructose, or *d*-mannose, the same equilibrium is reached, and an equilibrium mixture is obtained containing *d*-glucose, *d*-fructose, *d*-mannose, α- and β-*d*-glutose, and *d*-pseudo fructose. These reciprocal relations are explained as being due to the conversion of the sugar into an enol form common to all.



(The last four carbon atoms have identical configuration.)

From the enol form or ethylene oxide form, it can be easily seen that either glucose, fructose, or mannose can be regenerated. It is also possible for fructose to yield a second enolic form:



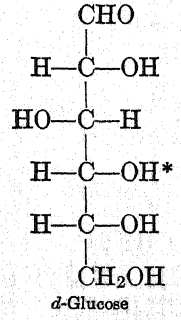
[18] Lobry de Bruyn, C. A., Action des alcalis dilués sur les hydrates de carbone I, *Rec. trav. chim.*, 14: 156–165 (1895); Lobry de Bruyn, C. A., and Ekenstein, W. A. van, II. Transformation reciproque des uns dans les autres des sucres glucose, fructose et mannose, *ibid.*, 14: 203–216 (1895); *cf.* also, Ekenstein, W. A. van, and Blanksma, J. J., Transformation du *l*-gulose et du *l*-idose en *l*-sorbose, *ibid.*, 27: 1–4 (1908).

Either glucose or fructose can be readily partially converted into glutose by treatment with solutions of calcium hydroxide at 75° to 80° in the absence of air.[19] (Two kilos of invert sugar dissolved in 8 liters of water, to which the $Ca(OH)_2$ derived from 30 grams of CaO had been added; the flask was then evacuated and heated three to four hours at 75° to 80°. The fermentable sugars were then removed by addition of 300 grams of starch-free yeast and the residual glutose syrup concentrated and purified.) The diabetic organism does not utilize glutose, and the tolerance of man and animals for glutose is extremely low. In most instances, the greater part is excreted unchanged. Insulin shock is not affected by the injection of glutose. It does not form a hexose phosphate; neither is it fermentable by yeast.

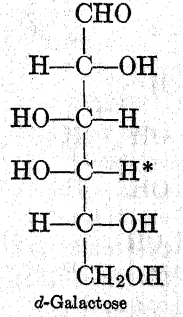
The Lobry de Bruyn transformation takes place[20] in disodium phosphate solutions, the aldo sugars being partially converted into ketoses, and *vice versa*. *d*-Glutose is formed from *d*-glucose in good yield. Apparently the Lobry de Bruyn transformation does not represent a true equilibrium, for in the experiments noted *d*-glutose was not transformed back into glucose and fructose when it alone was treated with disodium phosphate solutions. Spoehr even goes so far as to raise the question whether we actually know the true formula for *d*-glutose.

In a similar manner the *d*-galactose series yields an equilibrium mixture containing *d*-galactose, *d*-talose, *d*-tagatose, *d*-sorbose, and α- and β-*d*-galtose.

The sugars of the *glucose* series have never been converted (*in vitro*) into those of the *galactose* series, because the difference between the two series lies in the configuration on carbon-4. Apparently the rearrangements involve only carbon-1, -2, and -3, the molecule being most reactive at the carbonyl end.



d-Glucose



d-Galactose

It is interesting to compare the ratio of the amounts of sugars found in nature and in the Lobry de Bruyn equilibrium mixture. *d*-Glucose

[19] Benedict, E. M., Dakin, H. D., and West, R., On Glutose and Its Biochemical Behavior, *J. Biol. Chem.*, 68: 1–14 (1926).

[20] Spoehr, H. A., and Wilbur, P. C., The Effect of Disodium Phosphate on *d*-Glucose and *d*-Fructose, *J. Biol. Chem.*, 69: 421–434 (1926).

and *d*-fructose are the most important of the naturally occurring hexoses and are found in greatest abundance. *d*-Mannose, *d*-galactose, and *l*-sorbose are the only others found at all commonly in nature. Nef found ketoses and aldoses in about equal proportion in his equilibrium mixture. In the glucose series, *d*-glucose and *d*-mannose were the only aldoses present, and they were in a ratio of 5 glucose : 1 mannose. In the galactose series, *d*-galactose accounted for 90 per cent of the aldoses which were present. Accordingly, the nature and proportion of the sugars present in the equilibrium mixture closely approach the nature and proportion of the sugars occurring in plant products.

Wolfrom and Lewis,[21] in a study of the alkali-carbohydrate equilibrium, used extremely mild methods for the production of the equilibrium mixture. Under such conditions, they found that *d*-glucose was converted by the dilute alkali into a mixture of 63.4 per cent *d*-glucose, 30.9 per cent *d*-fructose, and 2.4 per cent *d*-mannose, leaving a balance of only 3.3 per cent which they state is composed of "non-sugar substances, probably saccharinic acids." They then studied the equilibrium mixture obtained from tetramethylglucose in the presence of dilute alkali. In this instance, no keto sugars were formed, the equilibrium mixture containing an equal amount of tetramethylglucose and tetramethylmannose. Figure 144 shows the interconversion of tetramethylmannose and tetramethylglucose into the equilibrium mixture consisting of 50 per cent tetramethylmannose and 50 per cent tetramethyl glucose. They note that the same equilibrium is reached from both directions and that the rate of conversion of tetramethylglucose is the same as the rate of the conversion of the tetramethylmannose. They suggest that in tetramethylglucose only the 1.2 dienol can form and that the enol formation is not due to hydration followed by a loss of water, but is actually due to the shifting of a hydrogen atom.

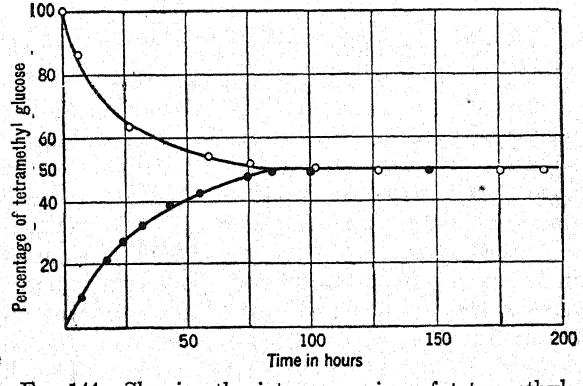
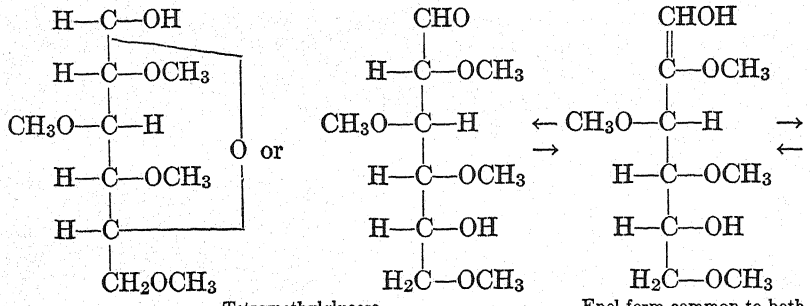


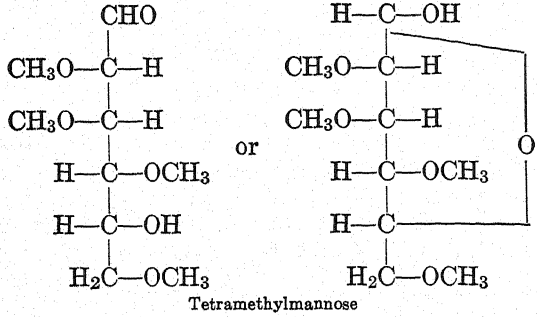
FIG. 144.—Showing the interconversion of tetramethyl mannose (solid dots) and tetramethylglucose (open circles). (Data of Wolfrom and Lewis.)

[21] Wolfrom, M. L., and Lewis, W. L., The Reactivity of the Methylated Sugars. II. The Action of Dilute Alkali on Tetramethyl Glucose, *J. Am. Chem. Soc.*, 50: 837–854 (1928).



Tetramethylglucose

Enol form common to both tetramethylglucose and tetramethylmannose



Tetramethylmannose

B. In the higher concentrations of alkali, oxidation results. Mathews [22] placed 2 grams of sugar in a 400-cc. flask and added 50 cc. of 0.4 *N* KOH, shaking the mixture constantly, and measured, by means of changes in pressure, the oxygen which was absorbed by the solution. He found that all sugars oxidized spontaneously in the presence of air. Glucose, galactose, maltose, and lactose oxidized at approximately the same rate but less rapidily than fructose. In certain experiments he allowed sugars to stand for a time in contact with alkali in an oxygen-free atmosphere, *e.g.*, the air replaced by hydrogen. When oxygen was admitted to such solutions, it was rapidly absorbed, indicating that the rearrangement produced by the alkali changes the rate with which sugars combine with oxygen. The theory for this change in rate assumes that the glucose molecule has been broken up into actively reducing fragments. Figure 145 shows the rate of absorption of oxygen for solutions of glucose, fructose, and galactose, in the presence of alkali. It also shows the curve where glucose was treated with alkali in the absence of oxygen, and oxygen was later admitted to the system.

[22] Mathews, A. P., Spontaneous Oxidation of the Sugars, *J. Biol. Chem.*, 6: 3–20 (1909).

Nef,[23] as a result of his studies, presented a theory for the oxidation of the sugars in alkaline solution. He assumed that in the presence of alkali the hexoses form 1-2, 2-3, and 3-4 dienols, and that beside giving a mixture of six sugars, these dienols split at the double

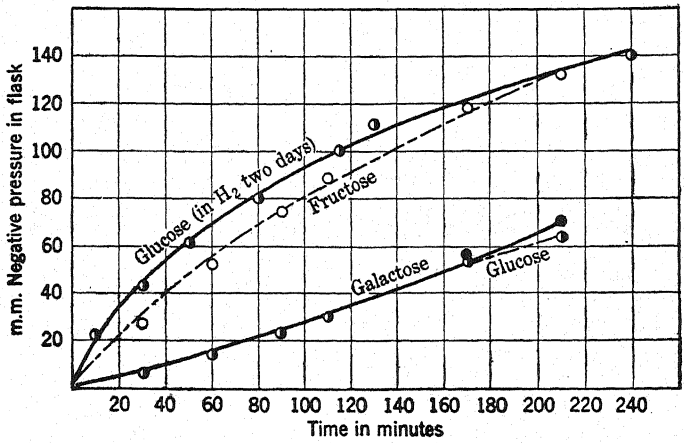
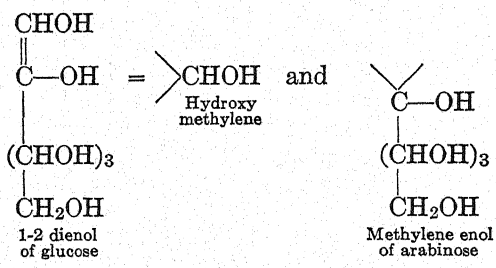


FIG. 145.—Showing the rate of absorption of oxygen by solutions of glucose, fructose, and galactose dissolved in 0.4 *N* KOH solutions. Also the increased reactivity of a glucose-NaOH solution after standing for two days in a hydrogen atmosphere. (Data of Mathews.)

linkage into a mixture of various carbohydrate-like compounds containing 1, 2, 3, 4, and 5 carbon atoms, the rupture at the double bond yielding radicals which contain *divalent carbon.*

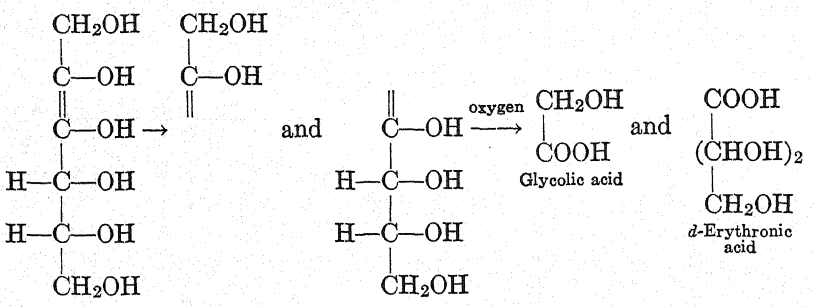
Thus, the 1-2 dienol of glucose would give rise to hydroxymethylene (divalent carbon) and the methylene enol of arabinose (again divalent



carbon). Inasmuch as divalent carbon should be an exceedingly reactive form, these radicals would absorb oxygen to yield formic acid from the hydroxymethylene, and *d*-arabonic acid (with some *d*-ribonic acid) from the methylene enol of arabinose.

[23] Nef. J. U., Dissociationsvorgänge in der Zuckergruppe, *Ann.*, 357: 214–312 (1907); *Ann.*, 403: 204–383 (1914).

The 2-3 dienol gives the methylene enol of a diose and of *d*-erythrose,



which on exposure to oxygen will yield glycolic acid and erythronic acid (some *l*-threonic acid is also formed). *d*-Erythronic acid is the chief optically active trihydroxybutyric acid found among the oxidation products of *d*-glucose when air or hydrogen peroxide is used as oxidizing agent in the presence of six equivalents of sodium hydroxide.

The 3–4 dienols break up into two molecules of the methylene enol of glyceric aldehyde, $CH_2OH—CHOH—COH$, which oxidize to *d*- and *l*-glyceric acid.

Under ordinary conditions (alkali only) none of the six sugars present in the equilibrium mixture oxidize to C_6 acids. This, however, takes place [24] when Fehling's solution is used.

The factors which must be controlled in an exact study of the reactions of sugars in alkaline solutions are (at least): (1) the concentration of alkali (or salt); (2) the oxidation potential (*i.e.*, air, H_2O_2, $KMnO_4$, $Cu(OH)_2$, AgO, etc.); (3) temperature; and (4) time. The earlier work in this field was carried out without exact control of hydrogen-ion concentration and without exact measurements of oxidation potential by the more modern technic. Evans [25] and his students have been repeating and extending the observations of Nef, using more exact physicochemical control of the systems, and have shown the importance of all the four items noted above, as governing the nature of the reaction.

[24] Glattfeld, J. W. E., On the Oxidation of *d*-Glucose in Alkaline Solution by Air as well as by Hydrogen Peroxide, *Am. Chem. J.*, 50: 135–157 (1913).

[25] Evans, W. L., *et al.*, The Mechanism of Carbohydrate Oxidation, I–XXIII, *J. Am. Chem. Soc.*, 47: 3085–3098, 3098–3101, 3102–3105 (1925); 48: 2665–2677, 2678–2681, 2703–2714 (1926); 50: 486–492, 1496–1503, 2267–2285, 2543–2556 (1928); 52: 294–307, 3680–3685, 4065–4069 (1930); 53: 4384–4400 (1931); 54: 698–704 (1932); 55: 4957–4963 (1933); 57: 200–202 (1935); *J. Org. Chem.*, 1: 1–16 (1936); *J. Am. Chem. Soc.*, 58: 797–800, 1661–1665, 1890–1894, 1950–1952, 2388–2395 (1936); and Evans, W. L., The Mechanism of Carbohydrate Oxidation, *Chem. Rev.*, 6: 281–315 (1929).

The different sugars show a wide variation in the proportion of the products formed. Carbon dioxide, formic acid, and oxalic acid may be obtained. Alkaline silver oxide is perhaps the most drastic of all the oxidizing agents, for by its use the sugars yield almost exclusively carbonic, formic, and oxalic acids.

While the sugars are very weak acids and poorly ionized, their salts are highly ionized. This increases the concentration of the sugar ions in solution, and it is thought that the greater reactivity is due to these unstable sugar ions. The carbon atoms decrease in activity as their distance from the carbonyl group increases.

Nef has shown that glucose in the presence of sodium hydroxide reacts to yield an equilibrium mixture containing at least 93 different compounds, and concluded that (1) the initial fragments undergo molecular rearrangement to form more stable compounds, (2) they may react with each other, one being oxidized and the other reduced, (3) they may combine or polymerize, and (4) they may react with other substances, such as oxygen, to form acids, etc. If we include synthetic steps, glucose and sodium hydroxide will yield, according to theory, no less than 116 compounds. Positive evidence has been obtained for at least 93.

Because of this ease of dissociation and decomposition with the liberation of energy, the sugars can be used as energy sources by living organisms, both under anaerobic and aerobic conditions. In vital processes, *e.g.*, in the cell, the sugar is not in a pure aqueous solution but in a colloidal system in the presence of considerable amounts of inorganic salts and reactive chemical groups. Such conditions lead to a dissociation of the sugar molecule, and the ions in turn break up, liberating energy. Spoehr believes that enzymes are simply special means for bringing about this dissociation. If the enzymes formed salts with the sugars which later dissociated, we would have the reactive sugar ions set free to decompose. Undoubtedly rearrangement and decomposition of the molecule precede metabolic utilization of the sugars.

Spoehr and Smith [26] note that sodium ferropyrophosphate acts as a catalyst for the oxidation of carbohydrates, so that they are easily oxidized by atmospheric oxygen. Palit and Dhar [27] report the oxidation of carbohydrates in an essentially neutral solution in the presence of cerous hydroxide or ferrous hydroxide as catalysts. They believe that the oxidation of carbohydrates in the cell is catalyzed by certain

[26] Spoehr, H. A., and Smith, J. H. C., Studies on Atmospheric Oxidation. I. The Oxidation of Glucose and Related Substances in the Presence of Sodium Ferro-Pyrophosphate, *J. Am. Chem. Soc.*, 48: 236–248 (1926); II. The Kinetics of the Oxidation with Sodium Ferro-Pyrophosphate, *ibid.*, 48: 107–112 (1926).

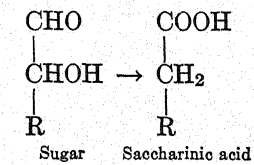
[27] Palit, C. C., and Dhar, N. R., Catalytic and Induced Oxidation of Some Carbohydrates, Uric Acid, and Inorganic Substances, *J. Phys. Chem.*, 30: 939–953 (1926).

of the cell constituents, so as to bring about the formation of the reactive sugar radicals.

Power and Upson,[28] using 100 grams of *d*-glucose dissolved in 10 liters of saturated calcium hydroxide, found that 95 grams of the glucose was oxidized by drawing air through the solution for 236 hours. They obtained 4.87 grams of carbon dioxide, 23.1 grams of volatile acids, mainly formic acid, and 84.9 grams of non-volatile acids, in which mixture they identified *d*-arabonic acid, *d*-erythronic acid, *l*-glyceric acid, glycolic acid, and oxalic acid (4.1 grams). Fructose under similar conditions gave a somewhat lower yield of carbon dioxide (4.28 grams) and somewhat more oxalic acid (9.1 grams). Glucic acid, $CH(OH)=CH—COOH$, the enolized semi-aldehyde of malonic acid, has been isolated[29] from a glucose-calcium hydroxide oxidation mixture.

Witzemann[30] studied the oxidation of glucose with hydrogen peroxide in the presence of disodium phosphate and states that disodium phosphate is the only chemical substance known to be generally necessary to the life of organisms which is able to catalyze the quantitative oxidation of glucose to carbon dioxide and water. Under laboratory conditions he was able to bring about the complete oxidation of glucose with hydrogen peroxide in the presence of disodium phosphate. We know that hydrogen peroxide is a product of cell activity and that phosphates are present in all cells, so that possibly the reactions which he studied in the laboratory are analogous to reactions taking place in living organisms. Later he[31] found that the reactions in the presence of guanidine are very similar to the reactions in the presence of sodium and potassium hydroxides.

Saccharinic acids are formed by internal oxidation and reduction of aldomonosaccharides, and possess the same empirical formula as the sugar from which they are derived. They are ordinarily formed[32] in



Sugar Saccharinic acid

[28] Power, M. H., and Upson, F. W., The Oxidation of *d*-Glucose by Air in Calcium Hydroxide Solution, *J. Am. Chem. Soc.*, 48: 195–202 (1926).

[29] Nelson, E. K., and Browne, C. A., The Properties and the Chemical Constitution of Glucic Acid, *J. Am. Chem. Soc.*, 51: 830–836 (1929).

[30] Witzemann, E. J., Disodium Phosphate as a Catalyst for the Quantitative Oxidation of Glucose to Carbon Dioxide with Hydrogen Peroxide, *J. Biol. Chem.*, 45: 1–22 (1920).

[31] Witzemann, E. J., The Action of Guanidine upon Glucose in the Presence and Absence of Oxygen, *J. Am. Chem. Soc.*, 46: 790–794 (1924).

[32] Nef, J. U., Dissoziationsvorgänge in der Zuckergruppe, *Ann.* 376: 1–119 (1910).

alkaline solution, in the absence of an oxidizing agent. A C_4-saccharinic acid has been isolated [33] from the oxidation products of maltose in the presence of alkali and air. This is the first instance where the alkaline oxidation of a sugar yielded a saccharinic acid. The acid which was isolated was *d-l*-2-3-dioxybutyric acid.

ACTIVE GLUCOSE.—Evidence has been accumulating for a considerable period of time that in the complex equilibrium of sugar solutions there are certain constituents which, even though they may be present only in traces, show very great reactivity. Thus, glucose solutions appear to contain an especially reactive form of glucose which has been designated as "active glucose." The evidence for this constituent has been reviewed by Levene.[34] Using oxidation-reduction potential technics, Clifton and Ort [35] come to the conclusion that a small but very definite amount of a powerful reductant or "active glucose" exists in a glucose solution, probably in the approximate ratio of 1 part of active glucose to 266,000 parts of ordinary glucose. They suggest that this may be either the free aldehyde form, or perhaps the oxide ring has opened with the formation of a free radical. Later Roepke and Ort [36] studied the rate of formation of active glucose as a function of temperature and hydrogen-ion concentration. The rate of formation of active glucose as a function of *p*H was found to obey the equation

$$\log V = 1.116\ p\mathrm{H} + C \qquad (183)$$

where V = the rate of formation of the active reductant,
C = a constant depending upon the concentration of the glucose, the concentration of buffer salts present, and the temperature.

The rate at *p*H 13 is 1,780,000 times greater than that at *p*H 7.4, so that the amount of active reductant formed in 1 minute at *p*H 13 would require approximately 3.3 years for formation at *p*H 7.4, all other conditions remaining constant. Similarly, the rate of formation of the reductant is enormously accelerated by temperature. At 100° C. the rate is approximately 24,000,000 times the rate of formation at 18° C., so that the amount of active reductant formed in 1 minute at 100° C. would require approximately 45 years for its formation at

[33] Glattfeld, J. W. E., and Miller, G. E., The C_4-Saccharinic Acids. I. The Resolution of *dl*-2,3-Dioxybutyric Acid into the Optically-Active Components. The Derivatives of These Acids, *J. Am. Chem. Soc.*, 42: 2314–2321 (1920).

[34] Levene, P. A., Active Glucose, *Chem. Rev.*, 5: 1–16 (1928).

[35] Clifton, C. E., and Ort, J. M, Active Glucose, *J. Phys. Chem.*, 34: 855–862 (1930).

[36] Roepke, M. H., and Ort, J. M., The Rates of Formation of the Active Reductants of Several Sugars, *J. Phys. Chem.*, 35: 3596–3611 (1931).

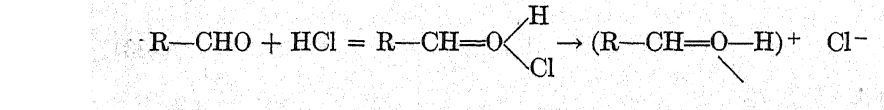
18° C., all other conditions remaining constant. The temperature equation is

$$\log V = 0.09T + C_2 \qquad (184)$$

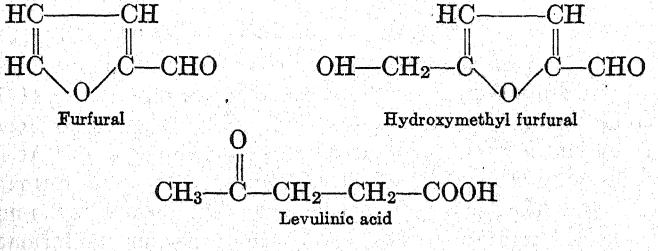
where C_2 = another constant under a given set of experimental conditions. They found that the concentration of the glucose in solution affects the rate of formation of the reductant. A 60 per cent glucose solution increases the rate of formation 2.7 times what the rate would be in a 1 per cent solution. Glucose, mannose, maltose, and lactose behave more or less alike so far as the rates of reactions are concerned. Galactose did not obey the rate of reaction equations for the other sugars.

THE ACTION OF ACIDS ON CARBOHYDRATES.—The sugar molecule is the most stable in neutral solution and in the absence of metallic salts. It is the least stable in the form of a salt. The decomposition reactions usually proceed at a slower rate in acid solution than under alkaline conditions, and some of the primary decomposition products formed in acid solutions can be readily isolated.

Apparently the first reaction is that of a salt formation, the formation of oxonium salts on the carbonyl group (tetravalent oxygen) and subsequent ionization.



Rearrangement then takes place with a loss of water and the formation of furfural from pentoses and of levulinic acid and formic acid or of hydroxymethyl furfural from hexoses.



Other products may be formed, depending upon the experimental conditions.

The formation of furfural from pentoses is of importance from the standpoint of laboratory technic, inasmuch as this reaction is utilized for the quantitative determination of pentoses and pentose-containing materials. Numerous methods have been devised for the quantitative production of the theoretical amount of furfural from the pentose ma-

terial and for the subsequent quantitative estimation of the furfural which was formed. The usual method employed is to boil the carbohydrate-containing material with 12 per cent hydrochloric acid, collecting the furfural in the distillate and determining the furfural either by precipitating it as the relatively insoluble furfural-phloroglucide or by estimating the furfural directly by either some titration method or by an appropriate colorimetric method. Inasmuch as hexoses form a small amount of hydroxymethyl furfural and rhamnose yields methyl furfural, the older methods for the estimation of the true pentoses have always yielded somewhat uncertain results, owing to the possible contamination of the furfural, which was formed, with these other similar compounds.

THE LABORATORY SYNTHESIS OF MONOSACCHARIDES.—The synthesis of *d*-glucose by Emil Fischer was one of the outstanding achievements of the organic chemist.

1. As already noted, the first synthesis of a sugar was that by Butlerow in 1861. He treated formaldehyde with saturated calcium hydroxide solution and secured a sweet syrup having the properties of a carbohydrate. This sugar, Loew named "formose."

2. Fischer and Tafel (1887–1889) treated acrolein dibromide with barium hydroxide and secured a sweet syrup containing two products which they called "α- and β-acrose."

$$CH_2Br—CHBr—CHO + Ba(OH)_2 = C_6H_{12}O_6 + 2BaBr_2$$

3. Fischer later used glycerose as the starting point for a sugar synthesis. Crude glycerose is a mixture of glyceric aldehyde and dihydroxyacetone. These apparently undergo aldol condensation with the formation of a ketose which has the same formula as fructose:

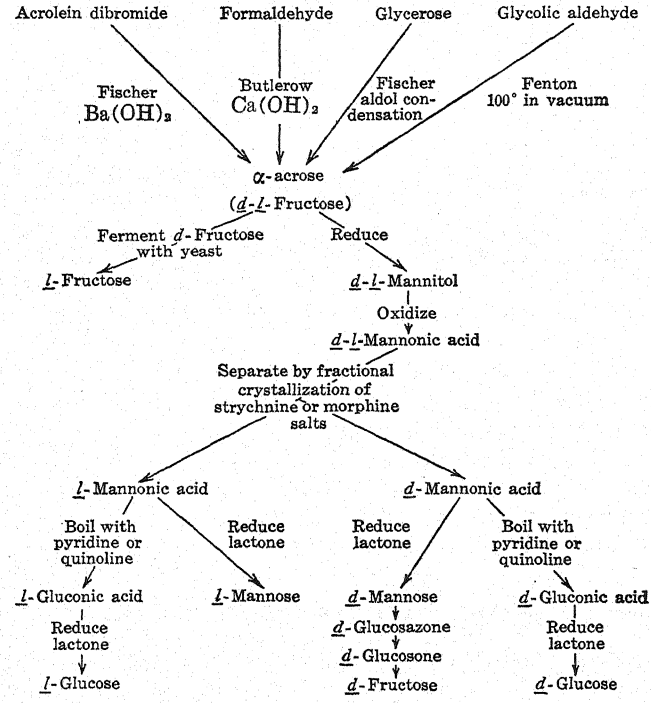
$$\begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CHO \\ \text{Glyceric aldehyde} \end{array} + \begin{array}{l} CH_2OH \\ | \\ C{=}O \\ | \\ CH_2OH \\ \text{Dihydroxy-acetone} \end{array} \xrightarrow[\text{condensation}]{\text{Aldol}} \underset{\alpha\text{- and } \beta\text{-Acrose}}{CH_2OH—(CHOH)_3—CO—CH_2OH}$$

This was again a mixture of α- and β-acrose, and its osazone differed from that of glucose only by being optically inactive.

4. Fenton, in 1897, heated glycolic aldehyde in a vacuum at 100° and secured a solid transparent "glass." This again contained α-acrose.

From his studies Fischer concluded that α-acrose was *d-l*-fructose, and he succeeded in proving this and in synthesizing from the synthetic sugar, *l*-fructose, *d*-fructose, *l*-mannose, *d*-mannose, *l*-glucose,

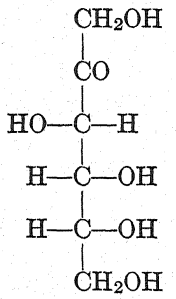
d-glucose, *l*-gulose, and *l*-idose.[37] His scheme of synthesis was as follows:



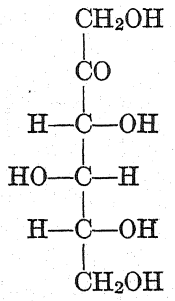
Thus the structure of α-acrose was proved. It was not until 1924, however, that we had any knowledge as to the nature of β-acrose. β-Acrose was shown[38] to be *d*-*l*-sorbose, although there is present a considerable amount of a keto pentose which is called "araboketose." The space formulas for these sugars are:

[37] Fischer describes these last two compounds as *d*-glucose and *d*-idose, inasmuch as they were derived from *d*-saccharic acid. However, the carboxyl group of carbon-1 was first reduced to yield *l*-gulonic acid, and the carboxyl group on carbon-6 was then reduced to an aldehyde to yield *l*-gulose. The aldehyde group, therefore appears on what was carbon-6 of the original glucose, thus accounting for the inversion from the *d*- to the *l*- sugar series. Similarly the gulonic acid derived by the reduction of saccharic acid was converted into *l*-idonic acid by boiling with pyridine or quinoline, and the lactone of this acid on reduction yielded *l*-idose, again with the aldehyde group on what had been carbon-6 of the original glucose.

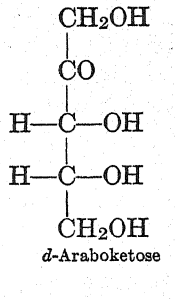
[38] Küster, W., and Schoder, F., Über das Entstehen von Sorbose bei der Kondensation des Formaldehyds, *Z. physiol. Chem.*, 141: 110–131 (1924).



d-Fructose



d-Sorbose



d-Araboketose

So far as the author is aware, galactose has not been synthesized in the chemical laboratory, although it has been used as the starting point for the synthesis of talose. *d*-Galactose is oxidized to *d*-galactonic acid. This, when boiled with pyridine or quinoline, is converted into *d*-talomucic acid, and the lactone of this acid on reduction yields *d*-talose.

THE CONVERSION OF GLUCOSE TO GALACTOSE.—No one has as yet been able to convert glucose into galactose *in vitro*, but *in vivo* this conversion apparently takes place readily in the mammary gland where lactose is formed. Glucose injected into the blood of a lactating animal causes lactosuria; when the mammary gland is not active, glucosuria results. In the animal body galactose is converted into glucose, although it is a slow process,[39] and when appreciable quantities of galactose are fed, some galactose is excreted in the urine. It appears probable that the synthesis of galactose in the animal body may be through the breaking down of glucose to lactic acid and the formation of galactose from the lactic acid so formed, since this appears to be the mechanism of the synthesis [40] of lactose in the mammary gland.

FERMENTATION OF PENTOSES AND HEXOSES.—This phase of carbohydrate study is extremely interesting because by this reaction energy is released and valuable industrial products are formed. It likewise involves problems of chemical configuration and biological specificity.[41] The transformations are caused by yeasts, bacteria, molds, etc., and are, of course, in the last analysis dependent upon enzymes.

Pasteur treated *d-l*-tartaric acid with *Penicillium glaucum* and found that the *d*-form was utilized by the organism, leaving the *l*-form unattacked. If ammonium lactate were present, the *l*-form only was

[39] Harding, V. J., and van Nostrand, F. H., Variations in Blood and Urinary Sugar after the Ingestion of Galactose, *J. Biol. Chem.*, 85: 765–778 (1930).

[40] Peterson, W. E., *et al*, unpublished data—personal communication.

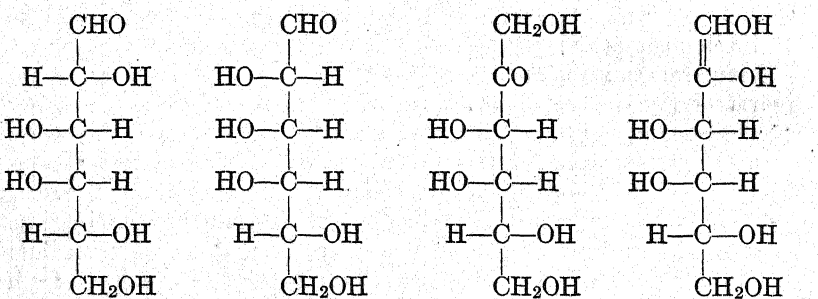
[41] For those who may be interested in a more extended discussion of the intermediate products of yeast fermentation, *cf.* Nord, F. F., Chemical Processes in Fermentations, *Chem. Rev.*, 3: 41–79 (1926).

utilized. *Penicillium glaucum* uses only the *l*-form of mandelic acid (phenylglycolic acid, C_6H_5—CHOH—COOH), whereas the yeast, *Saccharomyces ellipsoideus*, uses only the *d*-form.

Yeast ferments only *d*-glucose, *d*-mannose, *d*-fructose, and *d*-galactose. These are the *naturally occurring sugars*. The synthetic *l*-forms remain unaltered. *d*-Glucose, *d*-fructose, and *d*-mannose are fermented with the same ease, and the rate of fermentation of these three sugars has the same temperature coefficient. This is explained by the fact that they have a similar space configuration. It has already been pointed out that they are interconvertible and that they have the same enolic form.

It may be assumed that one of the necessary steps in fermentation is enolization. The enol forms, then breaking down to ethyl alcohol and carbon dioxide, the break being at the double bond of the enol formula. Further proof that enolization is probably the deciding factor lies in the fact that the α- and β-methylglucosides and gluconic acids are not fermentable. Their terminal carbon atom differs from the configuration of the terminal carbon of glucose, and they cannot enolize.

Galactose ferments with difficulty; some yeasts do not ferment it at all. The fermentation curve, with temperature as a variable, is decidedly different from the curve for glucose, fructose, and mannose. The fermentation of galactose, therefore, is probably a different reaction. Possibly a different enzyme is involved in the enolization of galactose. Rather interestingly, however, talose and tagatose, which have the same enol form as galactose, are not fermentable by any known yeast.

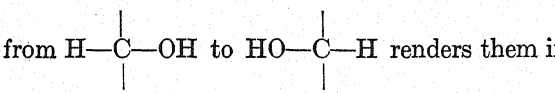


It is interesting to note that in talose the upper two carbon atoms have the same configuration as in mannose and the lower four the same configuration as in galactose; nevertheless talose is not fermentable, again a striking example of biological specificity.

There is a very close relationship between structure and the enzymes which cause fermentation. None of the ordinary yeasts ferment

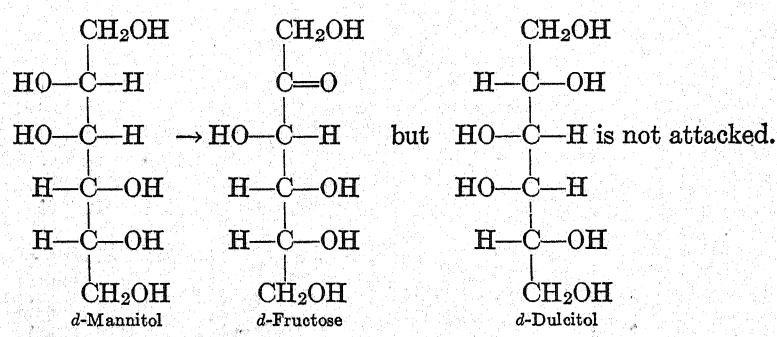
the pentoses. Apparently only the three-carbon sugars are readily fermentable, *i.e.*, trioses, hexoses, and nonoses. (The available data state that the one known nonose is fermentable. This point needs to be verified.)

The same specificity has already been noted in the case of the glucosides where the β-glucosides are hydrolyzed by emulsin and the α-glucosides by maltase. However, the α-methyl-*d*-xyloside, which is identical with α-methyl-*d*-glucoside except for dropping one carbon atom, is not hydrolyzed by these enzymes. Shortening the chain by one carbon atom spoils the harmony of enzyme action. Galactosides are not hydrolyzed by these enzymes; *l*-glucosides are not hydrolyzed by any enzyme; *d*-mannosides are not hydrolyzed (a single shifting



Fischer has likened the close relationship between enzyme and substrate to a lock and key. Only a specific key will open a specific lock! However, changing an —OH group to an —OR group does not spoil the harmony. Lactose is hydrolyzed by lactase which also hydrolyzes β-galactosides. Therefore, we know that lactose is a glucose-β-galactoside.

THE ACTION OF MICROORGANISMS ON SUGARS.—*Bacterium xylinum* oxidizes aldoses to monobasic acids and oxidizes certain alcohols to ketoses, but apparently oxidizes only such alcohols as have a =CHOH group adjacent to a $—CH_2OH$ group and when the —OH of the adjacent =CHOH group is of a configuration similar to the =CHOH group being oxidized. For example,



Within the last few years we have seen a large chemical industry develop, making use of biological organisms in order to produce desirable organic compounds from carbohydrates. The organisms used include yeasts, bacteria, and the fungi. Of course, certain of these reactions have been used commercially for many years, such as alcohol

by yeast fermentation, vinegar (acetic acid) by the acetic bacteria, and lactic acid by the lactic acid bacillus. We have now, however, controlled fermentations which will produce acetone, *n*-butyl alcohol, amyl alcohol, various aliphatic aldehydes, formic acid, citric acid, etc. In many instances, pentoses may be utilized, including the carbohydrates in such waste products as corncobs (*cf.* Fred and Peterson, *et al.*,[42] and Gabriel [43]). This particular field of study offers attractive possibilities for the development of commercial sources of many organic compounds.

Certain of the fungi are not specific in their fermentation requirements. The flax wilt organism, *Fusarium lini*, will ferment [44] to ethyl alcohol and carbon dioxide almost any naturally occurring sugar, either pentose or hexose, with equal ease, and when the carbohydrate is exhausted, will utilize the ethyl alcohol, oxidizing it to carbon dioxide and water.

Pentose-fermenting organisms are widely distributed in nature. The lactic and acetic fermentations of sauerkraut and silage are largely due to their activity. In silage the pentoses, sucrose, glucose, and fructose are readily fermented, but the starch is not attacked and remains unaltered in the silage.[45]

Bacteria and fungi in particular are capable in many instances of

[42] Peterson, W. H., and Fred, E. B., The Role of Pentose-Fermenting Bacteria in the Production of Corn Silage, *J. Biol. Chem.*, 41: 181–186 (1920); Fermentation of Fructose by Lactobacillus Pentoaceticus, N. Sp., *ibid.*, 41: 431–450 (1920); The Fermentation of Glucose, Galactose, and Mannose by Lactobacillus Pentoaceticus, N. Sp., *ibid.*, 42: 273–287 (1920); The Production of Acetaldehyde by Certain Pentose-Fermenting Bacteria, *ibid.*, 44: 29–46 (1920); Fred, E. B., Peterson, W. H., and Davenport, A., Fermentation Characteristics of Certain Pentose-Destroying Bacteria, *ibid.*, 42: 175–189 (1920); Fred, E. B., Peterson, W. H., and Anderson, J. A., The Relation of Lactic Acid Bacteria to Corn Silage, *ibid.*, 46: 319–327 (1921); Peterson, W. H., Fred, E. B., and Verhulst, J. H., The Destruction of Pentosans in the Formation of Silage, *ibid.*, 46: 329–338 (1921); Fred, E. B., and Peterson, W. H., Fermentation Process for the Production of Acetic and Lactic Acids from Corncobs, *Ind. Eng. Chem.*, 13: 211–213 (1921); Peterson, W. H., Fred, E. B., and Verhulst, J. H., A Fermentation Process for the Production of Acetone, Alcohol, and Volatile Acids from Corncobs, *ibid.*, 13: 757–759 (1921).

[43] Gabriel, C. L., Butanol Fermentation Process, *Ind. Eng. Chem.*, 20: 1063–1067 (1928).

[44] Anderson, A. K., Biochemistry of Plant Diseases, The Biochemistry of *Fusarium lini* Bolley, Minnesota Studies in Plant Science, Studies in the Biological Sciences, No. 5, pp. 237–280 (1924); *cf.* also, Letcher, H., and Willaman, J. J., Biochemistry of Plant Diseases. VIII. Alcoholic Fermentation of *Fusarium lini*, *Phytopathology*, 16: 941–949 (1926); and White, Mollie G., and Willaman, J. J., Biochemistry of Plant Diseases. X. Fermentation of Pentoses by *Fusarium lini*, *Biochem. J.*, 22: 583–591 (1928); Biochemistry of Plant Diseases. XI. *Fusarium lini* and the Pyruvic Acid Theory of Alcoholic Fermentation, *ibid.*, 22: 592–595 (1928).

[45] Dox, A. W., and Yoder, L., Influence of Fermentation on the Starch Content of Experimental Silage, *J. Agr. Research*, 19: 173–179 (1920).

utilizing pentoses as their sole source of carbon, while other organisms may be wholly unable to utilize them. One of the organisms which does not attack pentoses is bakers' yeast, although it readily ferments the hexoses. Bakers' yeast may therefore be added to the mixture of hexoses and pentoses, and the hexoses fermented off, and then the pentoses remaining may be determined by Fehling's solution in the usual manner. If we know the original total reduction, the difference in the two determinations gives the hexose sugars which were fermented.[46] Spoehr used this method to follow the fate of carbohydrates in wheat seedlings, germinated and grown in the dark, and found that the pentoses decrease with the increase in age of the seedling.

The rare sugars are of value for the identification of bacteria.[47] Certain rare sugars are as specific for certain bacteria as are chemical reagents for certain organic compounds. Conversely, bacteria may be used as reagents to identify sugars. Harding *et al.*[48] have outlined a complete scheme for the analysis and identification of mixtures of glucose, fructose, mannose, galactose, sucrose, maltose, and lactose by utilizing specific yeasts and bacteria as chemical reagents.

THE TRANSFORMATION OF THE VARIOUS GROUPS OF SUGARS IN THE PLANT.—The transformation of the sugars into other groups within the plant appears to be independent of the primary process of photosynthesis. Spoehr[49] in his studies on the cacti, found that the carbohydrate ratios were controlled by at least two factors, *water content* and *temperature*. With low water content and high temperature, he found an increase in polysaccharides and pentosans and a decrease in monosaccharides. It may well be that the increase in pentosans was a response of the plants toward the elaboration of hydrophilic colloids to bind the water in the plant against the forces of transpiration and to build up an imbibition pressure to draw water from an already water-depleted soil. We have already noted the work of Newton, showing that native grasses of Alberta "bind" water in the semi-arid areas as the season advances.

Spoehr found that a high water content and a low temperature

[46] Davis, W. A., and Sawyer, G. C., The Estimation of Carbohydrates. IV. The Presence of Free Pentoses in Plant Extracts and the Influence of Other Sugars on Their Estimation, *J. Agr. Sci.*, 6: 406–412 (1914).

[47] Kendall, A. I., Bacteria as Chemical Reagents, *Chem. Met. Eng.*, 24: 56–60 (1921); Studies in Bacterial Metabolism, LXVI–LXIX, *J. Infectious Diseases*, 32: 355–383 (1923); Bacterial Metabolism, *Physiol. Rev.*, 3: 438–455 (1923); Bacteria and the Chemist, *Ind. Eng. Chem.*, 15: 1001–1002 (1923).

[48] Harding, V. J., Nicholson, T. F., Grant, G. A., Hern, G., and Downs, C. E., Preliminary Account of Analytical Methods for Individual Carbohydrates, *Trans. Roy. Soc. Can.*, Sect. V, 26: 33–43 (1932); Harding, V. J., and Nicholson, T. F., The Use of Some Microorganisms in Sugar Analysis, *Biochem. J.*, 27: 1082–1094 (1933).

[49] Spoehr, H. A., The Carbohydrate Economy of Cacti, Carnegie Institution of Washington, Publication 287 (1919).

reversed the conditions, causing an increase in monosaccharides and a decrease in polysaccharides and pentosans. It is to be regretted that similar experiments were not conducted for the other possible series, *i.e.*, high water—high temperature and low water—low temperature.

Apparently a fairly complex equilibrium is involved, probably controlled by enzyme action, and bound water content and temperature affect inversion and reversion of carbohydrates. Other workers have shown that at low temperatures the synthesis of starch from monosaccharides is interfered with and takes place only when the glucose content of the tissue fluids reaches a relatively high level. It has been observed, for example, that the needles of conifers accumulate starch during the actively growing season but that at the onset of winter temperatures the starch disappears, being converted into soluble sugars, chiefly glucose, so that in the northern portion of the United States the needles become quite starch-free early in the winter period. With the advent of the warmer days in the spring the starch reappears and the glucose content decreases.

The question of the relationship between temperature and carbohydrate transformation may become of economic importance. For example, in the storage of potatoes, at least three reactions appear to be involved:

1. The starch → sugar transformation;
2. The sugar → starch transformation;
3. The sugar → carbon dioxide and water transformation (the respiration process).

The rate of all these reactions is decreased by a lowering of the temperature, but the last two are decreased relatively more than the first one, so that below a certain point the tubers become "sweet." The critical temperature appears to lie somewhere between +4.5 and +6.0° C. If the storage temperature is lower than +4.5, soluble sugars increase, and at 0° C. approximately 3 per cent of sugar accumulates before equilibrium is reached.

Russow [50] was apparently the first person to describe the disappearance of starch in plant tissues under the influence of low temperatures. He noted that starch was present at the onset of winter in relatively large quantities in the woody stems of various plants, but that during the three winter months, December, January, and February, the starch had almost completely disappeared, being replaced by oils or fats. In controlled experiments he was able to demonstrate

[50] Russow, *Naturforscher-Gesellschaft bei der Universität Dorpat*, Jan., 1882, [*cf.* translation by Hamilton P. Traub, Dr. Russow on the Disappearance and Reappearance of Starch in the Bark of Woody Plants, *The Minnesota Horticulturist*, 55: 241–242 (1927)]; *cf.* also Müller-Thurgau, H., Über Zuckeranhäufung in Pflanzentheilen in Folge niederer Temperatur, *Landw. Jahrb.*, 11: 751–828 (1882).

a decrease in starch content when the woody tissue was placed under cold conditions, and a reappearance of the starch when such twigs were later raised to a higher temperature.

Others [51] have noted and studied these changes. Hopkins found that there was a marked acceleration in the rate of respiration of potato tubers at 0° C., such that for a very considerable period the rate of respiration was greater at this temperature than at +4.5°. This stimulation passed through a maximum and then decreased, but even after many days the rate of respiration was still greater than was that at +4.5°. Certain of his data are shown in Fig. 146.

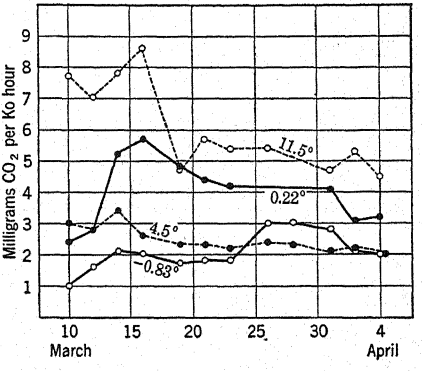


FIG. 146.—The respiration of tubers of russet rural potatoes at —0.83°, +0.22°, +4.5°, and +11.5° C., plotted against time. (Data of Hopkins.)

In a study of the rate of respiration with temperature, over the range from −0.83° to +11.5°, he noted a minimum point in the respiration curve at about +3.0°, as shown in Fig. 147.

In a study of the carbohydrate changes at various temperatures, Hopkins found that the sugar accumulation at 0° C. is at first slow, then rapidly increases, and finally slowly decreases. At +4.5°, the sugar content is nearly constant. At +1.17°, it increases fairly rapidly from the start, while at −0.83°, there is a gradual increase in total sugars but a decrease in reducing sugars. These data are shown in Fig. 148.

Hopkins suggests that the acceleration of respiration at 0° C. is dependent upon an increased concentration of sugar and that, up to a certain concentration, sugar increases respiration, and beyond that point inhibits it. Appleman and Smith [52] do not agree with this con-

[51] Appleman, C. O., Changes in Irish Potatoes During Storage, Bull. 167, Maryland Agr. Exp. Sta. (25th Ann. Rept.) pp. 327–334 (1912); Biochemical and Physiological Study of the Rest Period in the Tubers of *Solanum tuberosum*, *Botan. Gaz.*, 61: 265–294 (1916); *cf.* also Butler, O., The Significance of Sugar in the Tubers of *Solanum tuberosum*, *Bull. Torrey Botan. Club*, 40: 110–118 (1913); Bartholomew, E. T., A Pathological and Physiological Study of the Black Heart of Potato Tubers, *Centr. Bakt., Parasitenk.*, 43: 609–639 (1915); and Hopkins, E. F., Relation of Low Temperatures to Respiration and Carbohydrate Changes in Potato Tubers, *Botan. Gaz.*, 78: 311–325 (1924).

[52] Appleman, C. O., and Smith, C. L., Effect of Previous Cold Storage on the Respiration of Vegetables at Higher Temperatures, *J. Agr. Research*, 53: 557–580 (1936).

clusion, for they found that while those vegetables in which the percentage of starch was relatively high, and accordingly in which there was a rapid shifting of the carbohydrate equilibrium with temperature changes, were the ones that showed the greatest increase in initial respiratory rate, when transferred from a low to a higher temperature, nevertheless the respiration in these vegetables was, in general, lower than the respiratory intensity in a number of other vegetables having lower starch and lower sugar contents. They found no direct correlation between the content of either total sugars or reducing sugars and the respiratory rate of vegetables. Carrots and potatoes provided extremes of data.

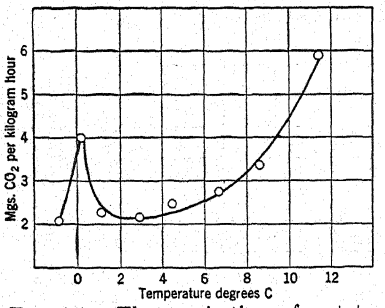


FIG. 147.—The respiration of potato tubers at various temperatures. (Data of Hopkins.)

Wolff [53] studied the sugar $\rightleftharpoons$ starch equilibrium in the potato over a somewhat wider range of temperatures than was covered by the studies of Hopkins. Certain of Wolff's data are shown in Fig. 149. He notes that the equilibrium is shifted not only by temperature changes but also by changes in the water content, and suggests that the area of sugar formation shown for the higher temperatures is in reality governed by changes in water relationships. Rather interestingly he notes that the pulp from macerated potatoes does not show an increased sugar content upon drying, such as is shown by the intact tuber, indicating rather definitely that gel structure or tuber structure influences the equilibrium.

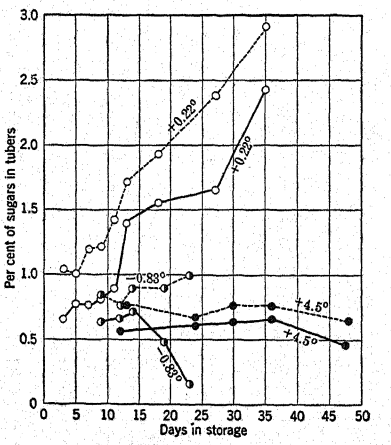


FIG. 148.—Showing carbohydrate changes in potato tubers held at low temperatures. Reducing sugars shown by solid lines, total sugars by broken lines. (Data of Hopkins.)

During growth the sweet potato tubers [54] contain very small amounts of sugar, so that in the summer they are "dry and tasteless." Following harvest, the starch $\rightarrow$ sugar transformation proceeds rapidly, first with the formation of

[53] Wolff, C. J., de, Die Saccharosebildung in Kartoffeln während des Trocknens. I., *Biochem. Z.*, 176: 225–245 (1926).

[54] Hasselbring, H., and Hawkins, L. A., Physiological Changes in Sweet Pota-

reducing sugars and then of sucrose. The rate of starch decrease and sugar increase follows closely Van't Hoff's rule of the relation of temperature to chemical reactions, *i.e.*, the rate approximately doubles for a 10° increase in temperature. It is highly probable that the transformation of starch to sugar in the sweet potato is not primarily dependent upon a temperature reaction, inasmuch as Gore[55] notes that sweet potatoes, when ripe, contain a very powerful diastase, sufficient to hydrolyze all of the starch to sugars. As a matter of fact, he utilized this reaction to prepare a syrup from sweet potatoes.

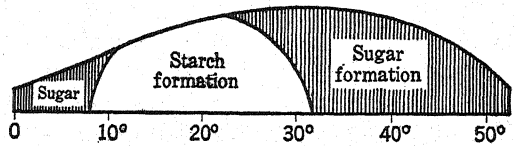


FIG. 149.—Showing diagrammatically the temperature effect on the sugar ⇌ starch equilibrium in the potato. (After de Wolff.)

The influence of cold as a stimulant to plant growth may well be associated with the transformation of starch to sugar. Coville[56] notes that many plants will not resume growth in continuously warm weather but will do so if subjected to a period of cold. In most of our trees and shrubs the reserve carbohydrate is starch, stored during a period of warm or hot weather. At the beginning of autumn the sapwood is gorged with this material, and as the process of chilling goes on, this reserve carbohydrate is transformed more and more into soluble sugars. As spring approaches, the starch practically disappears. Coville found that in the dormant blueberry wood, taken in the spring, the ratio of sugar to starch was about seven times what it was in a similar tissue in the autumn. Possibly a similar phenomenon is responsible for the "sugar flow" in the hard maple.

Coville also advances a theory to explain this paradoxical stimulating effect of cold upon plants. He suggests that the starch grains stored in the plant cells are at first separated by living, active cell membranes, from the enzymes which hydrolyze the starch, but that when the cell is chilled, the membranes undergo a change in permeability, so that the enzyme "leaks" out and hydrolysis results. If Coville's explanation is correct, anesthetics should have the same effect as low temperature.

THE ORIGIN OF THE PENTOSE SUGARS.—The pentose sugars occur in plants mainly in the form of polysaccharides—pentosans, for example,

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toes During Storage, *J. Agr. Research*, 3: 331–342 (1915); Behavior of Sweet Potatoes in the Ground, *ibid.*, 12: 9–17 (1918).

[55] Gore, H. C., Occurrence of Diastase in the Sweet Potato in Relation to the Preparation of Sweet Potato Syrup, *J. Biol. Chem.*, 44: 19–20 (1920).

[56] Coville, F. V., The Influence of Cold in Stimulating the Growth of Plants, *J. Agr. Research*, 20: 151–160 (1920).

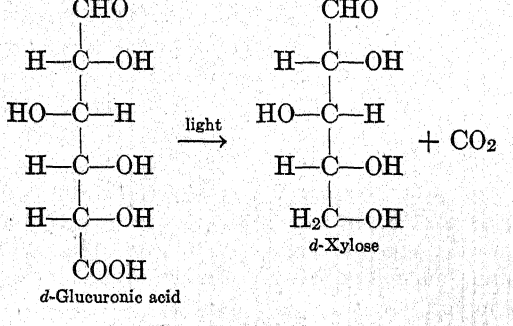
in the form of gums such as xylan or araban. Free pentose sugars have, however, been demonstrated in plants.[57]

Pentose sugars are apparently of great physiological importance, as evidenced by the fact that they are constant constituents of the nucleic acids. The question of the origin and mode of formation of the pentoses resolves itself into the question whether they are primary products of photosynthesis or whether they are derived from other sugars by metabolic processes.

In the aldose sugars carbon-1 is the reactive point and the reactions are mainly due to the carbonyl group. In the compound sugars and in the glycosides this group is combined in the molecule, so that it is taken out of the sphere of chemical reactivity. Under such conditions any chemical changes which occurred would take place on the $-CH_2OH$ group at the other end of the carbon chain. Such an oxidation would result in the formation of an aldehyde acid, such as glucuronic acid.

Spoehr found glucuronic acid to be present in cacti. This was an important observation, for it affords a rational theory for the formation of pentosans in plants. When a solution of glucuronic acid is exposed to sunlight, carbon dioxide is split off. This is a characteristic of many hydroxy acids. Malic acid ($COOH—CH_2—CH(OH)—COOH$), for example, loses two molecules of carbon dioxide and forms ethyl alcohol.

The natural pentoses belong to both the *l*-series and the *d*-series of sugars. *d*-Glucuronic acid on the loss of carbon dioxide forms *d*-xylose.

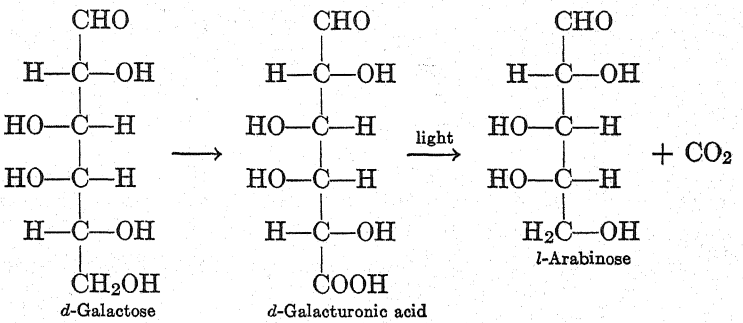


d-Glucuronic acid — *d*-Xylose

Direct oxidation of the aldehyde group and loss of carbon dioxide would have formed *d*-arabinose. It is a striking fact that *d*-glucose is usually associated in nature with *d*-xylose. Similarly *d*-galactose forms *d*-galacturonic acid which loses carbon dioxide to form *l*-arabinose. Here again the *d*-galactose is associated in nature with *l*-arabi-

[57] Davis, W. A., and Sawyer, G. C., The Estimation of Carbohydrates. IV. The Presence of Free Pentoses in Plant Extracts and the Influence of Other Sugars on Their Estimation, *J. Agr. Sci.*, 6: 406–412 (1914).

nose and not with *d*-lyxose, as would be the case if the aldehyde group were oxidized and carbon dioxide were lost.



Bacteria in certain instances are also able to reduce *d*-glucuronic acid to *d*-xylose.

We have already noted that mannuronic acid occurs in alginic acid [58] as a polymer anhydride. In this polymer all the carboxyl groups are free and all the aldehyde groups are conjugated. Mannuronic acid by decarboxylation should give rise to *d*-lyxose. It would be of very great interest to carefully investigate the nature of pentosans in the algae containing alginic acid to see whether or not lyxans might be present. Alginic acid is associated in nature with 4–5 per cent of mannitol. The acid itself is very hydrophilic; it swells but it does not disperse into a mucilage.

We also noted the *l*-ketoxylose which occurs in certain cases of pentosuria. This compound is fairly well metabolized by the dog,[59] whereas *d*-xylose is not. It is a true *l*-series sugar. It bears the same relationship to *d*-xylose that *l*-sorbose bears to *d*-glucose, *i.e.*, if *d*-xylose were reduced to *d*-xylitol, and then this pentahydroxy alcohol underwent oxidation on what had been carbon-4 of the *d*-xylose, *l*-xyloketose would result in the same way that *l*-sorbose results from the oxidation of carbon-5 of *d*-sorbitol. This seems to be the only way to account for the occurrence of *l*-xyloketose in the animal body. Other forms of pentosuria are characterized by the presence of *l*-arabinose.

THE SIGNIFICANCE OF PENTOSANS IN PLANTS.—Pentosans are the principal constituents of plant gums and mucilages, where they often occur in association with uronic acids. They are highly hydrophilic (German, "Pflanzenschleim") and undoubtedly play a major role in the water relations of certain plants, especially the succulent desert

[58] Nelson, W. L., and Cretcher, L. H., The Alginic Acid from *Macrocystis pyrifera*, *J. Am. Chem. Soc.*, 51: 1914–1922 (1929).

[59] Greenwald, I., The Possible Significance of *l*-Xyloketose (Urine Pentose) in Normal Metabolism, *J. Biol. Chem.*, 91: 731–734 (1931).

plants, such as the cacti. This highly hydrophilic property is not a property of the hexose polysaccharides. The relation of hydrophilic colloids in plants to water-retaining capacity has already been discussed under colloids.

Occurrence of Monosaccharides in Nature.—Glucose and fructose are the only two monosaccharides to occur commonly in the free state. Sorbose, a ketohexose, and La Forge's two ketoheptoses (*d*-mannoketoheptose and sedoheptose) occur rarely. All the other naturally occurring sugars are almost invariably found in some combined form, either as compound sugars, polysaccharides, glycosides, or in the form of their corresponding alcohols.

Mannose occurs as mannosans, anhydride-like condensation products which may be hydrolyzed to mannose by acids. The best source for the preparation of mannose [60] is vegetable ivory, the endosperm of the seed of the tagua palm, *Phytelephas macrocarpa*. Mannose is the true aldehyde of the alcohol mannitol, and may be obtained by oxidizing the alcohol. Fischer first prepared it in this manner and later found that it occurred in nature. The only reports of its occurrence in the animal kingdom are in connection with the trisaccharide, aminoglucose-dimannose, the carbohydrate constituent of egg albumin, serum albumin, and serum globulin.

Galactose occurs as a constituent of milk sugar (glucose-galactose) and of raffinose (galactose-glucose-fructose) and as the polymer, "galactan," in gums, mucilages, and pectins. It is usually associated in galactans with arabinose or xylose. Its occurrence as a galactoside is rare, except in milk sugar, and in the "saponins" where it is encountered rather frequently. It may be conveniently prepared [61] from the wood of the western larch.

Fructose, the sweetest of all the sugars, occurs free in most fruit juices. Honey is largely "invert sugar" and owes its superior sweetness to the fructose content. Its polysaccharide is inulin, the reserve carbohydrate in the tubers of the dahlia, chicory, and the Jerusalem artichoke. Various "inulides" also occur, usually associated with the true inulin.

The specific rotary power of a fructose solution changes markedly with temperature, becoming *less negative* as the temperature increases. At 87.3° C. it becomes equal to and opposite to the optical rotation of glucose. Therefore, at 87.3° C. invert sugar has a zero rotation. Fructose can be separated from solution as the calcium salt. The relative sweetening power of the sugars, on the basis of sucrose = 100, is shown in Table LXXIII.

[60] Hudson, C. S., and Sawyer, H. L., The Preparation of Pure Crystalline Mannose and a Study of Its Mutarotation, *J. Am. Chem. Soc.*, 39: 470–478 (1917).

[61] Schorger, A. W., and Smith, D. F., The Galactan of *Larix occidentalis*, *J. Ind. Eng. Chem.*, 8: 494–499 (1916).

TABLE LXXIII

RELATIVE SWEETNESS OF THE VARIOUS SUGARS
(DATA OF BIESTER, WOOD, AND WAHLIN) [62]

Sucrose	100.0	Maltose	32.5
Fructose	173.3	Rhamnose	32.5
Invert Sugar	130.0	Galactose	32.1
Glucose	74.3	Raffinose	22.6
Xylose	40.0	Lactose	16.0

[62] Biester, Alice, Wood, M. W., and Wahlin, C. S., Carbohydrate Studies I. The Relative Sweetness of Pure Sugars, *Am. J. Physiol.*, 73: 387–396 (1925).

CHAPTER XXV

THE HIGHER SUGARS

The higher sugars may be divided into the disaccharides, trisaccharides, tetrasaccharides, etc., formed by the condensation of two, three, four, or more molecules of monosaccharides with the elimination of one molecule of water less than the total number of monosaccharide molecules which are involved.

The determination of the structure of a compound sugar is extremely difficult, as may be appreciated from the fact that there has been until very recently uncertainty regarding the actual structure of sucrose. The determination of structure involves: (1) the nature of the constituent sugars; (2) the stereochemical forms (α or β); (3) the location of the alcohol groups involved in the linkage; and (4) the position of the internal oxygen rings in the constituent monosaccharides.

The method used in studying the structure has been to introduce into the molecule non-hydrolyzable residues (*e.g.*, methylate or acetylate all the alcohol groups), and then hydrolyze the sugar into its component monosaccharide derivatives. From a study of the compounds thus obtained, in comparison with the synthetic products formed from the monosaccharides in other studies, it may be possible to locate the carbon atoms through which the linkage of the sugars takes place.

Buston and Schryver[1] isolated from cabbage leaves a new carbohydrate, having the formula, $C_3H_8O_4$, which is possibly a new type of disaccharide. From 90 kilos of cabbage leaves, 10.7 grams of the compound was obtained. It melts at 148°, is faintly sweet, is soluble in water, but insoluble in alcohol and other organic solvents. It forms a tribenzoyl derivative, does not form an osazone, does not reduce Fehling's solution, and is not hydrolyzed by acids. Its formula is apparently $CH_2OH—CHOH—O—CH_2OH$, and it may be looked upon as a disaccharide formed from glycolic aldehyde and formaldehyde, *i.e.*, a diose and a monose uniting to form a triose of an unusual type. Its interest to us is only from the theoretical standpoint, as a possible indication of a minor side reaction taking place in nature.

[1] Buston, H. W., and Schryver, S. B., The Isolation from Cabbage Leaves of a Carbohydrate, Hitherto Undescribed, Containing Three Carbon Atoms, *Biochem. J.*, 17: 470–472 (1923).

SUGARS OF THE FORMULA $C_{12}H_{22}O_{11}$.—These are analogous to simple glycosides which on hydrolysis yield two molecules of hexose sugars, *e.g.*, sucrose yields glucose and fructose.

Both mineral and organic acids hydrolyze disaccharides to the corresponding monosaccharides. The hydrogen-ion concentration is the important factor, and the hydrogen-ion concentration may be relatively low, as compared with many hydrolytic reactions, and still cause rapid hydrolysis.

This hydrolysis of disaccharides by acids was one of the early methods employed to measure the hydrogen-ion concentration of a solution. Likewise it was the first chemical reaction to be measured by physicochemical means, *i.e.*, by the change in optical rotation of the solution as hydrolysis progressed.

The disaccharides differ widely in the ease of hydrolysis by acids. Sucrose is rapidly hydrolyzed at 20° by normal sulfuric acid, while lactose needs prolonged heating at 80° to accomplish the same degree of hydrolysis. Armstrong states that the rates of hydrolysis are sucrose 1,240; maltose 1.27; lactose 1.00. Each of the naturally occurring disaccharides is hydrolyzed by its own specific enzyme, *e.g.*, sucrase, maltase, lactase, etc. The enzyme hydrolysis is usually more rapid and more complete than acid hydrolysis. Commercial concentrated preparations of sucrase (invertase) are used in preparing "invert syrup" from sucrose.

The disaccharides may be classified into two groups:

I. The reducing disaccharides have one or more of the aldehyde groups potentially functional.

II. The non-reducing disaccharides have no potentially functional aldehyde group.

I. Reducing Disaccharides

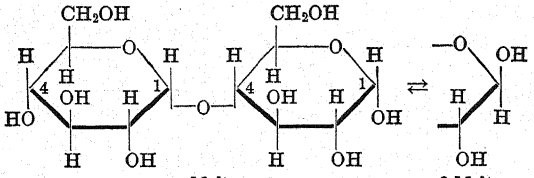
Maltose	Glucopyranose-4-α-glucopyranoside
Lactose	Glucopyranose-4-β-galactopyranoside
Isolactose	Glucose-?-galactoside
Melibiose	Glucopyranose-6-α-galactopyranoside
Turanose	β-Fructofuranose-6-α-glucopyranoside
Gentiobiose	Glucopyranose-6-β-glucopyranoside
Cellobiose	Glucopyranose-4-β-glucopyranoside

II. Non-Reducing Disaccharides

Sucrose	α-Glucopyranose-1-β-fructofuranoside
Trehalose	α-Glucopyranose-1-α-glucopyranoside
Isotrehalose	β-Glucose-β-glucoside

The Reducing Disaccharides.—Maltose is the end product of the action of diastatic enzymes on starch and glycogen. It was first iso-

lated by De Saussure in 1819 from the products of the hydrolysis of starch. It reduces Fehling's solution, forms an osazone, is not hydrolyzed by sucrase, lactase, or emulsin, but is hydrolyzed by maltase to two molecules of glucose. Accordingly it can be regarded as a glucose-α-glucoside. Maltose has eight free hydroxyl groups, inasmuch as it forms an octaacetate. Haworth *et al.*[2] have shown that maltose possesses the following structure:



(Glucopyranose-4-α-glucopyranoside)

Pictet and Vogel[3] report the synthesis of maltose by heating an equimolecular mixture of α- and β-glucose in a vacuum at 160°. The mixture melts and then suddenly resolidifies with no change in temperature. The product which is formed appears to be a maltose hydrate, $C_{12}H_{22}O_{11} \cdot H_2O$, as shown by optical rotation, the melting point of the osazone, the acetate, and the nitrate, and by the optical rotation of the acetate and nitrate.

When starch is hydrolyzed by acid for the formation of glucose syrup or when *d*-glucose is acted upon by strong hydrochloric acid, a disaccharide isomeric with maltose is formed. This has been named "isomaltose" and has been considered as distinct from the other reducing disaccharides. It seems probable that the name isomaltose covers a mixture of disaccharides. Gentiobiose has been isolated from isomaltose by Berlin,[4] who notes that in the acid hydrolysis of starch a part of the hydrolysis product can be isolated as gentiobiose. Armstrong, however, objects to this being taken as evidence that isomaltose is gentiobiose, since he notes that gentiobiose may occur preformed in the starch molecule.

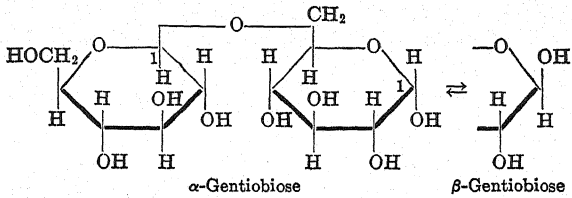
Gentiobiose, a reducing disaccharide, is not fermentable by yeast and differs from maltose in the fact that it is a β-glucoside rather than

[2] Cooper, C. J. A., Haworth, W. N., and Peat, S., The Constitution of the Disaccharides, X. Maltose, *J. Chem. Soc.*, 876–880 (1926); Haworth, W. N., and Peat, S., XI. Maltose, *ibid.*, 3094–3101 (1926).

[3] Pictet, A., and Vogel, H., Synthèse du maltose, *Helv. Chim. Acta*, 10: 588–593 (1927).

[4] Berlin, H., The Identity of Isomaltose with Gentiobiose, *J. Am. Chem. Soc.*, 48: 1107–1111 (1926); The Occurrence of Gentiobiose in the Products of the Commercial Hydrolysis of Corn Starch, *ibid.*, 48: 2627–2630 (1926).

an α-glucoside, and the linkage is on carbon-6 in gentiobiose rather than on carbon-4 as in maltose.



α-Gentiobiose (Glucopyranose-6-β-glucopyranoside) β-Gentiobiose

Gentiobiose is a constituent of a number of glycosides and is usually prepared from the trisaccharide, gentianose, which occurs in the roots of the gentian. It has been synthesized [5] by the action of emulsin on a concentrated glucose solution. Its structure was determined by Haworth *et al.*[6]

Lactose or milk sugar is the characteristic disaccharide of milk and is manufactured from milk whey. It reduces Fehling's solution, undergoes mutarotation, and forms an osazone. The potential aldehyde group belongs to the glucose radical, because upon oxidation and subsequent hydrolysis it yields galactose and gluconic acid.

Galactose occurs in the brain and in the medullary sheaths of the nerves as an important constituent of the glucolipids (*vide infra*). As already noted, we have no definite proof that any organ of the body, excepting the mammary gland, can transform glucose into galactose. It therefore appears that nature has made a provision for an adequate supply of galactose in the form of milk sugar during the early stages of brain development, and this consideration alone is sufficient to cast doubt upon the advisability of substituting sucrose for lactose in infant feeding. The fact that lactose occurs to such a large extent in milk whey makes this sugar potentially available in very large quantities.

Lactose is hydrolyzed by the specific enzyme lactase. It is not hydrolyzed by maltase, sucrase, or diastase. It differs markedly from the other sugars in the great ease with which it undergoes lactic acid and butyric acid fermentations.

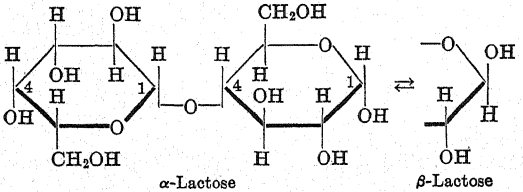
Pictet and Vogel [7] report the synthesis of lactose by heating, for half an hour in a 15-mm. vacuum at 175°, an equimolecular mixture of β-glucose and β-galactose in the presence of zinc chloride as a cata-

[5] Bourquelot, Em., Hérissey, H., and Coirre, J., Synthèse biochimique d'un sucre du groupe des hexobioses, le gentiobiose, *Compt. rend.*, 157: 732–734 (1913).

[6] Haworth, W. N., Loach, J. V., and Long, C. W., The Constitution of the Disaccharides. XVII. Maltose and Melibiose, *J. Chem. Soc.*, 3146–3155 (1927).

[7] Pictet, A., and Vogel, H., Synthèse du lactose, *Compt. rend.*, 185: 332–334 (1927); *Helv. Chim. Acta*, 11: 209–215 (1928).

lyst. The residue was freed from chlorides, acetylated, and the acetyl products saponified with sodium methylate, resulting in the isolation of a disaccharide identical with lactose in optical rotation, osazone, acetate, and nitrate. The structure of lactose has been shown by Haworth and Long.[8]



α-Lactose (Glucopyranose-4-β-galactopyranoside) ⇌ β-Lactose

Kunz and Hudson[9] report the conversion of lactose into a new disaccharide, neolactose, by treating lactose octaacetate with active aluminum chloride. In this way they obtained a chloracetyl derivative of lactose, and about 30 per cent of the chloracetyl derivative of a new sugar, neolactose. The new derivative was separated by fractional crystallization, and its properties and hydrolytic products determined. The galactose radical was unchanged, pure galactose being recovered upon the hydrolysis of neolactose. The *d*-glucose radical had been changed to *d*-altrose, as shown by the preparation of *d*-altronic acid and *d*-altrose phenylosazone, and by the isolation of *d*-altrose itself. Apparently both the α- and β-carbon atoms had been altered in this transformation.

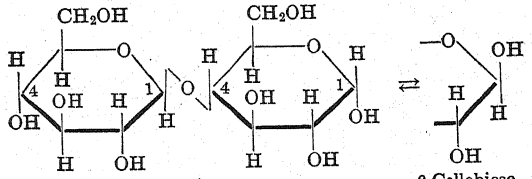
Cellobiose is a disaccharide obtained by the partial hydrolysis of either cotton or wood cellulose. It is the β-glucoside corresponding to maltose which is the α-glucoside. Its structure has been definitely established by Haworth *et al.*,[10] and it has been synthesized[11] by the condensation of acetobromoglucose with levoglucosan to give an intermediate product which, when acted on by 50 per cent sulfuric acid, was converted into cellobiose tetraacetate. Its only use at the present

[8] Haworth, W. N., and Long, C. W., The Constitution of the Disaccharides. XII. Lactose, *J. Chem. Soc.*, 544–548 (1927).

[9] Kunz, A., and Hudson, C. S., Relations between Rotatory Power and Structure in the Sugar Group. XV. Conversion of Lactose to Another Disaccharide, Neolactose. The Chloro-Hepta-Acetate and Two Octa-Acetates of Neolactose, *J. Am. Chem. Soc.*, 48: 1978–1984 (1926); XVII. The Structure of Neolactose, *ibid.*, 48: 2435–2439 (1926).

[10] Haworth, W. N., Long, C. W., and Plant, J. H. G., The Constitution of the Disaccharides. XVI. Cellobiose, *J. Chem. Soc.*, 2809–2814 (1927).

[11] Freudenberg, K., and Nagai, W., Die Synthese der Cellobiose. XXIII. Mitteilung über Acetonzucker und andere Verbindungen der Kohlenhydrate, *Ber.*, 66: 27–29 (1933).

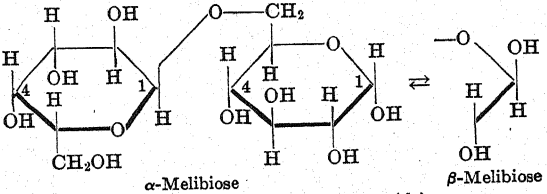


α-Cellobiose
(Glucopyranose-4-β-glucopyranoside)

β-Cellobiose

time is as a special sugar for bacterial culture media, where it is sometimes used to identify particular types of bacteria. It is utilized by *Aerobacter aerogenes*, but is not attacked by *Bacterium coli*, and is therefore specific in differentiating these two types of organisms. It is usually prepared [12] from cellobiose octaacetate.

Melibiose, a glucose-galactoside, is obtained together with fructose from the trisaccharide, raffinose. It differs from lactose in being an



α-Melibiose
(Glucopyranose-6-α-galactopyranoside)

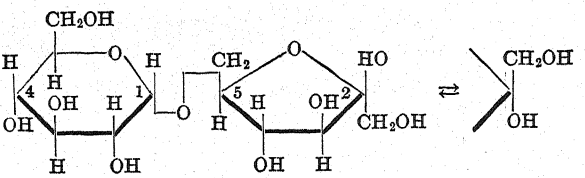
β-Melibiose

α-galactoside rather than a β-galactoside and in having the oxygen bridge attached to carbon-6 rather than carbon-4. Pictet and Vogel [13] report the synthesis of melibiose by converting glucose into a polymer, "diglucosan" by heating glucose *in vacuo* in the presence of zinc chloride. Galactose was similarly converted into "digalactosan." When a mixture of "diglucosan" and "digalactosan" was heated with zinc chloride, a glass-like solid was obtained, from which by appropriate technic a disaccharide was isolated, which they state to be identical in physical and chemical properties with melibiose.

Turanose was discovered by Alekhine, in 1889, as a partial hydrolysis product of the trisaccharide melezitose. It is difficultly hydrolyzable by acids. It is a β-fructofuranose-α-glucoside and has the structure:

[12] Peterson, F. C., and Spencer, C. C., A Note Concerning a New Method for the Production of Cellobiose from Cellobiose Octa-Acetate, *J. Am. Chem. Soc.*, 49: 2822–2825 (1927).

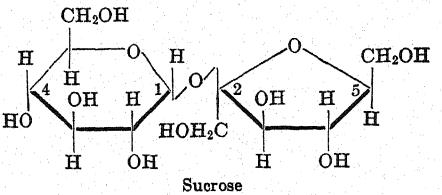
[13] Pictet, A., and Vogel, H., Synthèse du melibiose, *Helv. Chim. Acta*, 9: 806–809 (1926).



β-Turanose
(β-Fructofuranose-6-α-glucopyranoside)

α-Turanose

The Non-reducing Disaccharides.—The principal non-reducing disaccharide is *sucrose.* McOwan [14] and Haworth and Hirst [15] have apparently definitely settled the structure of sucrose, the glucose radical containing the δ-oxide ring, the fructose radical the γ-oxide ring, the union being that of a β-fructoside, as shown in the following formula:



Sucrose
(α-Glucopyranose-1-β-fructofuranoside)

The above formula explains (1) why sucrose forms an octaacetate, *i.e.*, there are eight free —OH groups, (2) why it does not form a phenylhydrazone, inasmuch as there are no "free" or "potential" aldehyde groups, (3) why it does not exhibit mutarotation, since it cannot form an aldehyde without hydrolysis, and (4) why it does not reduce Fehling's solution, inasmuch as it possesses no ketonic or aldehydic properties.

Many organic chemists have attempted the synthesis of sucrose. It appears, however, that none of these attempts have been successful in spite of occasional announcements of success. All attempts to confirm claims of success have failed.

Sucrose is very generally distributed throughout the plant kingdom, the commercial sugar being derived chiefly from the sugar cane and the sugar beet. The development of the sugar-beet industry originated because of the blockade of France during the Napoleonic wars. Napoleon offered a prize for a new source of sugar which would make France independent of the other nations for its sugar supply. At that time, the sugar beet contained only approximately 8 per cent

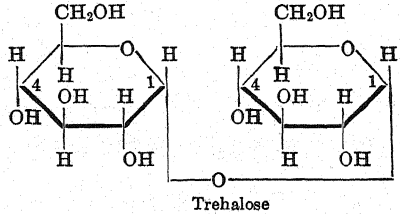
[14] McOwan, G., The Constitution of Sucrose, Pt. I. Oxidation of Tetramethyl γ-Fructose. Pt. II. Evidence Contributed by the Oxidation of *d*-Arabinose, *J. Chem. Soc.*, July, 1926, 1737–1750.

[15] Haworth, W. N., and Hirst, E. L., The Structure of Fructose, γ-Fructose, and Sucrose, *J. Chem. Soc.*, July, 1926, 1858–1868.

of sucrose. Through the science of plant breeding, aided by chemical analysis, strains of sugar beets have been developed so that at the present time beets which are grown for sugar average from 16 to 18 per cent of sucrose. In some instances, under exceptionally favorable conditions, the sucrose content may become as great as 23 per cent.

An interesting sucrose preparation is viscogen, prepared by saturating a 50 per cent solution of sucrose with calcium hydroxide and filtering off the excess of calcium hydroxide. This alkaline solution when added in small amounts to cream causes it to "whip" much more readily. No adequate explanation has been offered for the physical chemistry of this process.

Trehalose, an α-glucose-α-glucoside, is the only other important non-reducing disaccharide. It is found widely distributed in the fungi. Inasmuch as they are non-chlorophyll bearing, they form no starch and store trehalose as a reserve food supply. Trehalose does not show mutarotation and forms no osazone. It is not hydrolyzed by maltase, invertase, emulsin, or diastase, but is hydrolyzable by a special enzyme, trehalase, which occurs in yeast and in certain fungi, *e.g.*, *Aspergillus niger*. With acids it is hydrolyzable only with difficulty, showing a marked contrast to the behavior of sucrose.



Trehalose
(α-Glucopyranose-1-α-glucopyranoside)

SUGARS OF THE FORMULA $C_{18}H_{32}O_{16}$.—In the trisaccharides, which on hydrolysis yield three monosaccharides, we have again the reducing and non-reducing types.

I. Reducing Trisaccharides

Rhamninose	Galactose-rhamnose-rhamnoside
Robinose	Galactose-rhamnose-rhamnose (from the glycoside, robinine)
Mannotriose	Glucose-6 $\leftrightarrow$ 1-galactose-6 $\leftrightarrow$ 1-galactoside

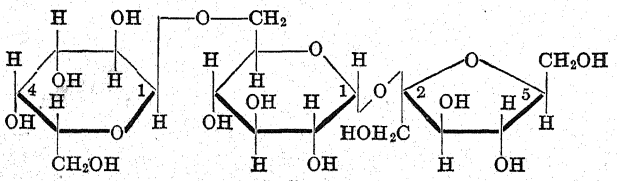
II. Non-reducing Trisaccharides

Raffinose	2-β-Fructofuranose-1-α-glucose-6-α-galactoside
Melezitose	1-α-Glucose-2-β-fructofuranose-6-α-glucoside
Gentianose	2-β-Fructofuranose-1-α-glucose-6-β-glucoside

Robinose was found by Charaux[16] in the glycoside robinine, obtained from the fresh flowers of *Robinia pseudoacacia* D.C. Charaux reports that the sugar reduces Fehling's solution in the cold, which would be an indication that at least one of the sugar radicals was present as the γ-sugar.

Raffinose is the best known of the trisaccharides. It occurs in appreciable amounts in the sugar beet, and special corrections have to be made to the readings of a polarimeter in order to determine accurately sucrose in the presence of raffinose. So far as its chemical reactions are concerned, it is quite similar to sucrose. Its[17] structure is that of two disaccharides combined in one molecule with the glucose radical in common. The disaccharides are melibiose and sucrose.

Galactose-O-glucose-O-fructose
(1) (2) (3)
Melibiose Sucrose
(4) (5)



Raffinose
(2-β-Fructofuranose-1-α-glucose-6-α-galactoside)

Here again we have a striking example of the specificity of enzyme action.

1. The strong mineral acids hydrolyze raffinose to its constituent monosaccharides, fructose, glucose, and galactose.

2. Weak acids (or a low hydrogen-ion concentration) hydrolyze only the sucrose part of the molecule, yielding fructose (3) and melibiose (4), as the hydrolytic products. Here we have the potential sucrose residue, showing the same ease of hydrolysis that characterizes sucrose.

3. Sucrase yields as hydrolytic products, fructose (3) and melibiose (4), *i.e.*, sucrase hydrolyzes only the sucrose residue.

4. Emulsin, in contrast to sucrase, hydrolyzes the melibiose portion of the molecule, yielding galactose (1) and sucrose (5).

[16] Charaux, C., Sur le dédoublement biochemique du Robinoside (Robinine).—, Robinose, nouveau triose, provenant de ce dédoublement, *Bull. soc. chim. biol.*, 8: 915–923 (1926).

[17] Haworth, W. N., Hirst, E. L., and Ruell, D. A., The Constitution of Raffinose, *J. Chem. Soc.*, 123: 3125–3131 (1923); Charlton, W., Haworth, W. N., and Hickinbottom, W. J., The Constitution of the Disaccharides. XIV. Melibiose and Its Relationship to Raffinose, *ibid.*, 1527–1536 (1927).

5. Bottom yeasts contain both melibiase and sucrase, so that they hydrolyze raffinose completely to fructose, glucose, and galactose.

In addition to being found in the sugar beet, raffinose is frequently found in the higher plants and in fungi. It has been reported to be present in certain invertebrates. Cottonseed meal contains about 8 per cent of raffinose and is the usual material from which this sugar is prepared.

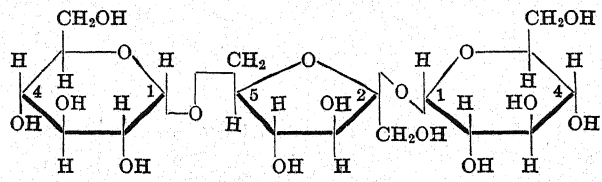
Raffinose is valueless as a food until after hydrolysis, and in man such hydrolysis as does take place is apparently due [18] to the bacterial action in the large intestine and not to any enzymes which we normally possess.

Gentianose is a trisaccharide which bears the same relationship to gentiobiose that raffinose bears to melibiose, *i.e.*, it is a β-glucosidosucrose [19] in the same way that raffinose is an α-galactosidosucrose.

Glucose + glucose + fructose
(1) (2) (3)
Gentiobiose (4) Sucrose (5)

It is accordingly composed of two disaccharides, sucrose and gentiobiose, holding one glucose radical in common.

Melezitose is an exceedingly rare sugar. It is composed of three hexose molecules and can be hydrolyzed to yield glucose and the disaccharide turanose.



Melezitose
(1-α-Glucopyranose-2-β-fructofuranose-6-α-glucopyranoside)

Melezitose occurs in the sap of the larch, Douglas fir, scrub pine, etc., and under certain conditions, especially following attacks by sucking insects, these trees exude drops of sweet sap which harden into a more or less true "manna." In periods when nectar is scarce, bees may store this "honey dew" as a substitute for honey. This happened in the dry summer of 1917 in certain portions of the eastern

[18] Kuriyama, S., and Mendel, L. B., The Physiological Behavior of Raffinose, *J. Biol. Chem.*, 31: 125–147 (1917).

[19] Haworth, W. N., and Wylam, B., The Constitution of the Disaccharides. IX. Gentiobiose: Its Identity with Amygdalin Biose, *J. Chem. Soc.*, 123: 3120–3125 (1923).

United States when the clover blossoms quickly dried because of drought. The following winter, many bee colonies throughout this area died. The "honey" in these hives had crystallized. The Bureau of Chemistry of the United States Department of Agriculture undertook an investigation as to the cause of the death of the bees and found [20] the "honey" to contain very large quantities of melezitose. From this source they prepared several kilos of this rare sugar.

Apparently the honeybee can utilize melezitose to some extent,[21] inasmuch as both bees and larvae live much longer with an aqueous solution of pure melezitose than when only water is available. It is stated [22] that the order in which the honeybee can utilize the sugars is sucrose > fructose > maltose > melezitose > dextrose > trehalose > dextrin > galactose > lactose. Adult honeybees cannot utilize dextrin, galactose, or lactose. The larvae cannot utilize starch or glycogen.

SUGARS OF THE FORMULA $C_{24}H_{42}O_{21}$.—The only tetrasaccharide known is *stachyose*, isolated [23] from the tubers of *Stachys tuberifera*. On hydrolysis it yields one molecule of glucose, one molecule of fructose, and two molecules of galactose. On mild hydrolysis the molecule of glucose is split off, yielding a trisaccharide, mannotriose, containing one molecule of fructose and two molecules of galactose. Stachyose is apparently a raffinose-galactopyranoside: [24]

$$\overbrace{\text{Galactose-1-0-6-galactose-1-0-6-}\underbrace{\text{glucose-1-0-2-fructose}}_{\text{Sucrose}}}^{\text{Raffinose}}$$

The α and β linkages have not been fully established. It is nonreducing.

[20] Hudson, C. S., and Sherwood, S. F., The Occurrence of Melezitose in Honey, *J. Am. Chem. Soc.*, 42: 116–125 (1920); *cf.* also Wherry, E. T. The Crystallography of Melezitose, *ibid.*, 42: 125–128 (1920).

[21] Phillips, E. F., Die Verwertung der Melezitose durch die Honigbienen, *Z. Untersuch. Lebensm.*, 64: 383–389 (1932).

[22] Bertholf, L. M., The Utilization of Carbohydrates as Food by Honeybee Larvae, *J. Agr. Research*, 35: 429–452 (1927).

[23] Planta, A. von, and Schulze, E., Über ein neues krystallisirbares Kohlenhydrat, *Ber.* 23: 1692–1699 (1890).

[24] Onuki, M., Chemical Constitution of Stachyose. II, *J. Agr. Chem. Soc., Japan*, 9: 90–111 (1933).

CHAPTER XXVI

THE POLYSACCHARIDES

The polysaccharides are, in general, characterized by forming colloidal sols rather than true solutions. The micellar weight is invariably large, corresponding in some instances at least to the micellar weights of the proteins. Many of the polysaccharides are strongly hydrophilic. They may be classified as has already been indicated in Chapter XXII, or the following simple classification may be used.

I. Starches or hexosans
   - Glucosans
   - Fructosans
   - Mannosans
   - Galactosans
   - Amyloids, etc.

II. Gums or mucilages
   - Pentosans
   - Natural gums
   - Pectins
   - Plant mucilages

III. Polymerized glucosamine
   - Chitin

IV. Celluloses
   - Hemicelluloses
   - True celluloses
   - Compound celluloses

It is highly probable that very few of the groups mentioned above accurately express the nature of the material, for many of the polysaccharides contain non-carbohydrate radicals.

THE STARCHES OR HEXOSANS $(C_6H_{10}O_5)_x$.—These polysaccharides on hydrolysis yield hexoses. The chief hexosan is starch or amylum. It forms the reserve food material of most plants and is the source of energy for most plant embryos. Starch is to plants what the fats are to animals. Starch occurs in the cells in granules having characteristic striations. These striations and the size and shape of the granules are more or less characteristic of many plant forms and may be used as a

microsocopical means of identifying the biological origin[1] of the starch.

Amylases or diastase hydrolyze[2, 3, 4] the starch to dextrins, and these in turn to maltose, which is the end product of diastatic action. Acids or maltase continue the hydrolysis to glucose. Various degradation products have been given names, and it has been suggested that the following scheme represents the course of hydrolysis:

$$\text{Starch} \rightarrow \text{amylodextrin} \rightarrow \begin{matrix} \text{maltose} \\ + \\ \text{erythrodextrin} \end{matrix} \rightarrow \begin{matrix} \text{maltose} \\ + \\ \text{achroödextrin} \end{matrix} \rightarrow \text{maltose} \rightarrow \text{glucose}$$

The amylodextrins, erythrodextrins, and achroödextrins are differentiated by the color which they give with iodine, *i.e.*, blue → purple → red-brown. It has been suggested[5, 6] that these color changes do not necessarily mean anything more than a change in size of the starch particle and that in reality, instead of forming distinct compounds, the diastase simply peptizes the starch granules, the whole series of color reactions with iodine being colloidal phenomena depending on the degree of dispersion, in the same way that the color of colloidal gold sols changes with size of particle, being red at the smaller sizes and blue to black at the larger sizes.

The structure of starch is apparently a regular sequence of α-glucopyranose units joined in a glucosidal linkage, carbon-1 of one α-glucopyranose being joined to carbon-4 of another α-glucopyranose unit.

[1] Reichert, E. T., The Differentiation and Specificity of Starches in Relation to Genera, Species, etc., Stereochemistry Applied to Protoplasmic Processes and Products, and as a Strictly Scientific Basis for the Classification of Plants and Animals, Pts. I and II, Carnegie Institution of Washington, Pub. 173, Washington, D. C. (1913).

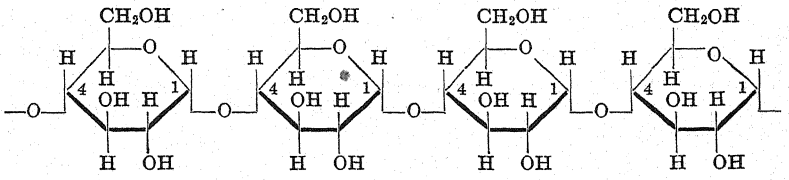
[2] Blake, J. C., On the Digestibility of Bread, I. Salivary Digestion *in Vitro*, *J. Am. Chem. Soc.*, 38: 1245–1260 (1916).

[3] Davis, W. A., The Distribution of Maltase in Plants. I. The Function of Maltase in Starch Degradation and Its Influence on the Amyloclastic Activity of Plant Materials, *Biochem. J.*, 10: 31–48 (1916).

[4] Daish, A. J., The Distribution of Maltase in Plants. II. The Presence of Maltase in Foliage Leaves. III. The Presence of Maltase in Germinated Barley, *Biochem. J.*, 10: 49–55, 56–76 (1916).

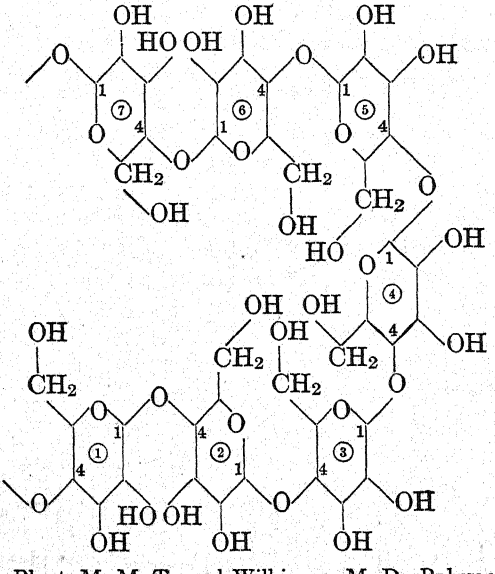
[5] Herzfeld, E., and Klinger, R., Zur Chemie der Polysaccharide, *Biochem. Z.*, 107: 268–294 (1920); *ibid.*, 112: 55–60 (1920).

[6] Stocks, H. B., Colloid Chemistry of Starch, Gums, Hemicelluloses, Albumin, Casein, Gluten, and Gelatine, First Report on Colloid Chemistry, *Brit. Assoc. Advancement Sci.*, pp. 46–78 (1917).



In such a chain those glucopyranose residues which occupy terminal positions on the chain would have four —OH groups free, whereas those which are not linked in the terminal position would have only three —OH groups free. When the ratio between trimethylglucose and tetramethylglucose was determined [7] for completely methylated starch, the conclusion was reached that the length of the starch molecule is from 24 to 30 glucose units as a minimum. This would give a minimum molecular weight of about 4,000.

Haworth notes that the above structural diagram of a segment of the starch granule may not actually represent the configuration of the entire chain, since the oxygen bridges linking the glucopyranose units together probably join the units at an angle which would permit the molecule to coil back upon itself in a series of closed loops, thus forming a more or less globular and compact structure where secondary valence forces of the exposed hydroxyl groups would tend to bind the coiled chain very closely together.



[7] Hirst, E. L., Plant, M. M. T., and Wilkinson, M. D., Polysaccharides. XIV. The Molecular Structure of Amylose and Amylopectin, *J. Chem. Soc.*, 2375–2383 (1932).

As might be anticipated with a product which has so much biochemical and commercial importance, an enormous literature concerning starch and starch derivatives has grown up. Much of this literature is exceedingly conflicting. In a great many instances the research has been conducted upon commercial samples of starches which have contained foreign materials, *e.g.*, proteins, mineral matter, etc., in small amounts, and since small amounts of such material markedly affect the properties of starch, particularly the colloidal properties, it is difficult to reconcile many of the statements which occur in the literature.

There has been prolonged controversy over the question of whether or not the starch granules are composed of a homogeneous chemical substance. The weight of evidence indicates that the starch granule contains two substances which from the physical standpoint at least are more or less distinct. One of these, which has come to be known as β-amylose (granulose), is readily dispersible in water to form sols of relatively low viscosity. The other substance, α-amylose or amylopectin, does not readily disperse in water, forms highly opalescent sols of high viscosity, and appears to be the "insoluble" fraction of the starch granule. For a long time it was believed that the amylopectin fraction formed the outer "hull" of the starch granule, acting more or less like a membrane with the β-amylose in the interior of the granule, and that when the starch granule was gelatinized by heat in the presence of water, the amylopectin hull swelled into a balloon-shaped sack still retaining the soluble β-amylose within the swollen granule. Other workers have suggested that the amylopectin is distributed more or less throughout the granule but that, since it forms a gel when the starch granule is gelatinized, the β-amylose is immeshed in the interstices of the α-amylose gel. α-Amylose has been separated from β-amylose by a variety of procedures including sedimentation, particularly with the aid of a centrifuge which tends to throw down the larger fragments of the amylopectin gel. Ultrafiltration technics likewise have been employed, using relatively porous ultrafilters through which clear sols of the β-amylose can pass. Probably the best technic for separation is that devised by Gruzewska [8] and used extensively by Samec [9] in his investigations, as well as by Taylor *et al.*[10] In this method the gelatinized starch paste is electrodialyzed. The β-amylose

[8] Samec, M., Studien über Pflanzenkolloide, IV. Die Verschiebungen des Phosphorgehaltes bei den Zustandsänderungen und dem diastatischen Abbau der Stärke, *Kolloid Beihefte*, 6: 23–54 (1914).

[9] Samec, M., Kolloidchemie der Stärke, Theodor Steinkopff, Dresden and Leipzig, (1927).

[10] Taylor, T. C., and Iddles, H. A., Separation of the Amyloses in Some Common Starches, *Ind. Eng. Chem.*, 18: 713–717 (1926); Taylor, T. C., and Walton, R. P., Characterization of Certain Starches and their Amyloses, *J. Am. Chem. Soc.*, 51: 3431–3440 (1929).

is essentially uncharged and remains in the solution. The α-amylose or amylopectin possesses a strong negative charge and migrates to the anode where it can be collected. Repeated electrodialysis completely purifies the amylopectin from the β-amylose.

Starches from various botanical sources differ widely in their content of α-amylose. According to Taylor, cornstarch contains from 11.5 to 15.6 per cent α-amylose, rice starch 15.9 to 17 per cent, tapioca starch 16.3 to 17.5 per cent, wheat starch 23.5 to 23.8 per cent, and potato starch only 1.7 to 1.9 per cent.

It has been known for a long time that starches, even when highly purified, contain traces of phosphoric acid, but until recently it has been assumed that this phosphoric acid was simply a part of the "ash" which is inevitably present in the preparations of most biological materials. However, the investigations of Taylor, Samec, etc., have demonstrated rather conclusively that the phosphoric acid is associated with the α-amylose or amylopectin fraction and that the β-amylose fraction is practically phosphorus free. More and more the theorem is being accepted that α-amylose represents a phosphoric acid ester of the starch molecule. The carbohydrate hydrolysis products of amylopectin are identical with those of β-amylose, and Haworth was unable to find any difference in the length of the amylopectin molecule as determined by the ratio of the yield of tetramethylglucose to trimethylglucose. That the phosphoric acid is a definite part of the starch molecule has apparently been finally proved by Posternak,[11] who separated from the hydrolytic products of potato starch glucose-6-phosphoric ester, identical with the Robison ester. The presence of phosphoric acid in amylopectin explains its migration in an electric field, for the phosphoric acid would be ionized, leaving a residual charge of sufficient magnitude on the polysaccharide micelle to make it migrate with appreciable velocity.

Koets[12] has proposed that amylopectin is not simply amylose phosphate but that it is a coacervate system in which the negatively charged amylose phosphate is coacervated with protein. He notes that all starches contain small amounts of nitrogen which again have been assumed to be impurities. He believes that this nitrogen represents protein attracted to the negatively charged centers where the phosphoric acid residues reside on the surface of the starch micelles. By deliberately inserting additional phosphoric acid groups into the starch

[11] Posternak, T., Sur le phosphore de la fécule de pommes de terre, *Compt. rend.*, 197: 1157–1158 (1933); and Sur un acide hexose-phosphorique obtenu par hydrolyse de la fécule, *ibid.*, 198: 506–507 (1934).

[12] Koets, P., Complex Coacervation of Amylophosphoric Acid and Proteins and Its Possible Bearing on the Problem of Amylopectin, *Proc. Acad. Sci. Amsterdam*, 38: 63–74 (1935); Coacervation of Amylophosphoric Acid and Proteins, *J. Phys. Chem.*, 40: 1191–1200 (1936).

molecule and preparing amylophosphoric acids with higher phosphorus contents he studied the coacervation of such systems with a number of proteins, and demonstrated that the coacervate theory of amylopectin had an experimental basis.

Haworth's formula and the 24 to 30 glucose units in the molecule do not take into account the possibility that phosphoric acid diesters may act as bridges binding two molecules together. Neither does it take into account the observation that gentiobiose is usually obtained in small quantities when starch is hydrolyzed with acid. Furthermore, Taylor *et al.*[13] have shown that oleic, linolic, and palmitic acids are invariably present as constituents of cornstarch, in quantities amounting to 0.5 to 0.6 per cent of the starch. Palmitic acid accounted for 24 per cent, oleic acid 40 per cent, and linolic acid 36 per cent of the total. Lehrman,[14] in a study of rice starch, isolated 14.75 grams of mixed fatty acids from 5 pounds of starch. These were identified as palmitic 36 per cent, oleic 35 per cent, linolic 29 per cent. These fatty acids occur in the α-amylose fraction apparently esterified on the hydroxyl groups.

The gelatinization of starch by heating with water has a very marked temperature coefficient. In general, no reaction will be observed until about 55° C. When starch is viewed under the microscope and this temperature has been reached, it will be noted that a few granules swell to a relatively enormous volume. As the temperature slightly increases, the number of swollen granules rapidly increases until all have swollen and a considerable number have burst. The point at which this transformation takes place is known as the gelation temperature or "thickening point" and has been reported as being more or less characteristic of the plant source from which the starch is derived. Dox and Roark,[15] however, in a study of 13 different varieties of maize, report a range in the gelation temperature from 64° to 71° C. Potato starch, as a rule, gelatinizes at a lower temperature than most other starch. Sago starch and rice starch, as a rule, require a higher temperature.

Grinding starch in a ball mill [16] causes it to disperse in water in a manner similar to "soluble starch." In a study of the gelatinization of

[13] Taylor, T. C., and Nelson, J. M., Fat Associated with Starch, *J. Am. Chem. Soc.*, 42: 1726–1738 (1920); Taylor, T. C., and Lehrman, L., The Unsaturated Fatty Acids Associated with Corn Starch, *ibid.*, 48: 1739–1743 (1926); Taylor, T. C., and Werntz, J. H., Properties of Corn Starch. Removal of Combined Fatty Acids, *ibid.*, 49: 1584–1588 (1927).

[14] Lehrman, L., The Fatty Acids Associated with Rice Starch, *J. Am. Chem. Soc.*, 51: 2185–2188 (1929).

[15] Dox, A. W., and Roark, G. W., Jr., The Determination of Gelatinization Temperatures of Starches by Means of an Electrically Heated Chamber on the Microscope Stage, *J. Am. Chem. Soc.*, 39: 742–745 (1917).

starch, Alsberg[16] finds that, contrary to most of the statements in the literature, starch granules when heated swell but *do not burst*, although the potato starch granules do disintegrate to some extent. He believes that it is because of this disintegration that gelatinized potato starch does not form such viscous sols as do the other starches. In investigating the swollen granules under the microscope, Alsberg noted that the granules appeared to be composed of a saclike structure filled with a fluid. When tannin was allowed to diffuse into such swollen granules, it formed particles which exhibited strong Brownian movement, indicating that the interior of the granules is either a sol or a solution, not a gel. When such starch pastes were ground, there was a pronounced decrease in viscosity, which Alsberg explains as being due to a rupture of the envelope of the starch granule, allowing the fluid interior to escape. When dry starch was ground in a ball mill, a similar disintegration took place, so that when the ground starch was later heated with water, a thick syrup was formed instead of a gel.

Alsberg notes three factors as being responsible for the gelatinization of starch by heat: (1) the ease with which the anatomical structure is softened by moist heat, (2) the inherent swelling power of the granule substance, and (3) the relation of the mass of the swelling substance to the surface area of the granule. The larger the granule, the greater is the mass of the swelling substance which exerts pressure upon a unit area of the granule surface. Therefore, a large granule will swell more rapidly than a small one, and it should swell sooner if the temperature is raised. Alsberg believes that there is no such physical constant as a "gelatinization temperature" but that, instead, there is only a "gelatinization range."

Alsberg and Griffing[17] report the crystallization of starch by autoclaving at 150° to 160° C. the clear solution prepared from starch which had been ground in a ball mill. Their starch crystals separated in needles which gave typical iodine reactions for starch, the needles being only slightly soluble in cold water but much more readily soluble in hot water.

We have already noted, when considering the viscosity of colloid systems, the decrease in viscosity which starch pastes undergo with time (*cf.* Fig. 11). This phenomenon is known as retrogression and is characteristic of all starch pastes, although starches from different botanical sources differ quantitatively in the effects which are observed. It seems unquestionably to be bound up with the degree of hydration of the starch. Probably in the initial hydration water is

[16] Alsberg, C. L., and Griffing, E. P., Effect of Fine Grinding upon Flour, *Cereal Chem.*, 2: 325–344 (1925); Alsberg, C. L., Studies upon Starch, *Ind. Eng. Chem.*, 18: 190–193 (1926).

[17] Alsberg, C. L., and Griffing, E. P., The Crystallization of Starch, *Proc., Soc. Exptl. Biol. Med.*, 23: 728–730 (1926).

attracted to the polar groups in the molecule. The chains of the starch molecules are thus forced apart in the same way that Miss Jordan Lloyd postulates that polypeptide chains are forced apart when proteins swell. On standing, syneresis sets in, the swollen carbohydrate gel becomes more or less dehydrated, and we say that the starch has retrograded. The retrogression of starch is the primary factor in the staling of bread. Stale bread may have the same water content as fresh bread, but the distribution of the water between the starch and protein determines whether it has the soft texture of fresh bread or the crumbly dry texture of stale bread.

In Table VI are shown calculations of the volume occupied by the hydrated starches from various botanical sources. No adequate explanation has as yet been proposed to account for the wide differences shown by the potato starch on the one hand and rice and maize starches on the other.

Starch may be gelatinized not only with heat but also in the cold in solutions of certain salts. The thiocyanates of sodium and potassium and sodium salicylate are particularly effective.[18] This gelatinization is not identical with the phenomenon of heat gelatinization, for when the volume occupied by the swollen granules is calculated by means of the Kunitz equation (8), the volume which is attained in cold gelatinization is very much greater than the volume attained in heat gelatinization. A study of the cold gelatinization process indicated that the taking up of water by the starch granule followed a typical adsorption isotherm as shown in Fig. 150, where the logarithms of the volume occupied by 1 gram of starch are plotted against the logarithms of the molarity of the peptizing solution. It will be noted that the

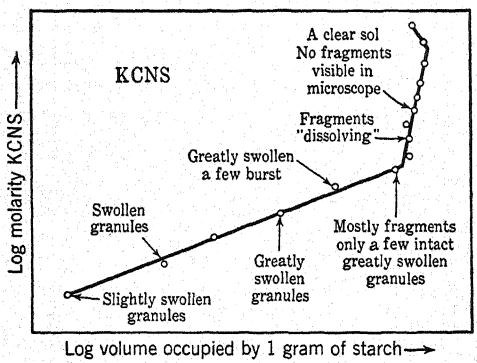


FIG. 150.—Showing the hydration behavior and accompanying optical properties of a wheat starch suspension peptized with various concencentrations of KCNS.

[18] Ostwald, Wo., and Frankel, G., Kinetische Studien über Stärke-Verkleisterung, I, *Kolloid Z.*, 43: 296–312 (1927); *cf.* also Gortner, R. A., The Hydration Capacity of Starch, *Cereal Chem.*, 10: 298–312 (1933), and Mangels, C. E., and Bailey, C. H., Relative Viscosities of Wheat Starches, *Ind. Eng. Chem.*, 25: 456–468 (1933), and Relation of Concentration to Action of Gelatinizing Agents on Starch, *J. Am. Chem. Soc.*, 55: 1981–1988 (1933).

logarithmic curve is made up of three straight lines. The first of the two intersecting straight lines is due to the swelling of the intact starch granules. The second straight line is due to the further peptization of the small micelles after the granules have burst. The third straight line appears to be a dehydration of the swollen starch micelles brought about by the withdrawing of water from the swollen granule by the higher concentrations of the peptizing agent.

The quality of starch, its sizing properties, and the viscosity of the starch sols and pastes differ widely from one botanical species to another, and for any single species are dependent upon the method of preparation of the starch sols or gels. Gelatinized starch shows marked hysteresis. Sago, tapioca, and cassava starches usually yield more gelatinous sols than the ordinary grain starches.

It is impracticable to go into any detail regarding the commercial preparation of starches or their industrial uses. The literature in this field is enormous. The reader is referred to the excellent survey which has been prepared under the editorship of Walton.[19]

The Mechanism of the Transformation of Glucose to Starch in the Plant.—The value of potassium as a fertilizer is at least in part due to its functioning importantly in starch formation. Potassium is apparently necessary in the reaction whereby glucose, the product of photosynthesis, is transformed into starch, the reserve food material. The nature of this reaction, however, is almost wholly unknown. From the standpoint of physical chemistry, it is essential that the plant transform the glucose into a higher polymer, such as starch, as a reserve food supply. So long as glucose is present in solution, the osmotic pressure within the cells must be at least proportional to the glucose concentration. When, however, the glucose is converted into the relatively insoluble and osmotically inert starch, a large reserve supply of food materials may be stored without unduly affecting the osmotic relations of the tissues.

Chapman [20] studied the enzymatic relationships in certain plants and suggests a mechanism for the transformation of glucose to starch within the cells. He notes, "The guard-cell starch of those plants which do not contain any other starch may be regarded as formed by a mechanism which later disappears. The snowdrop has no maltase. But starch cannot be formed from glucose without maltase (if enzymes are true catalysts), so that presumably in the young leaf, the whole apparatus—maltase, dextrinase, and amylase—required to convert glucose into starch is present in the guard-cells. Starch is formed, and afterwards the maltase disappears. Thus no more starch can be

[19] Walton, R. P., A Comprehensive Survey of Starch Chemistry, Vol. I, Chemical Catalog Company, New York (1928).

[20] Chapman, R. E., The Carbohydrate Enzymes of Some Starch-free Monocotyledons, *Biochem. J.*, 18: 1388–1400 (1924).

formed, but equally the starch already formed cannot be hydrolyzed further than maltose. The series of changes

$$\text{Starch} \rightleftarrows \text{Maltose}$$

under the influence of amylase and dextrinase would form an ideal mechanism for controlling the opening of the stomata if the reaction is

$$\text{Starch} \rightarrow \text{Maltose}$$

when the plant has plenty of water and transpiration is not too intense, and

$$\text{Starch} \leftarrow \text{Maltose}$$

when transpiration is high and water supplies are failing. But these are exactly the circumstances required, because the full equation is

$$\text{Starch} + \text{Water} \rightleftarrows \text{Maltose}$$

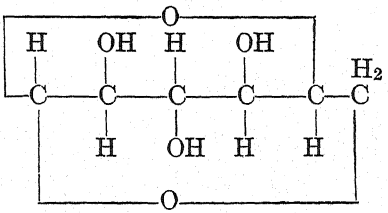
and a high water content in the leaf would cause the reaction to go to the right. The maltose thus formed would increase the soluble contents of the guard-cells, water would be taken in by osmosis until turgidity was reached, and the stomata would open. If water were lacking, the reaction would go to the left, maltose would be replaced by insoluble starch, the soluble contents of the guard-cells would be lessened, water would be lost by osmosis, the guard-cells would no longer be turgid, and the stomata would close. This system of amylase and dextrinase with no maltase might be described as a 'chemical appendix,' a relic of an earlier state, but in this case the appendix is still functional and useful. The data seem to indicate that there is only a small quantity of diastase in the snowdrop, which would be quite compatible with this theory.

"Similarly, the onion and the leek cannot form starch in the green leaf, because the complete starch-forming machinery is again lacking; the onion lacks dextrinase and the leek lacks amylase.

"The dock can form starch because it contains the full equipment of maltase, dextrinase, and amylase."

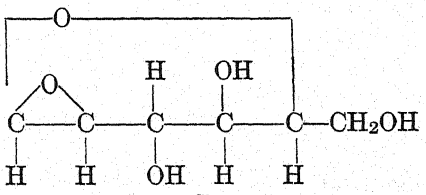
Dextrins (Sometimes called British Gum).—This is a collective name for a group of intermediate products formed by the partial hydrolysis of starch. They are distinguished from starch by being soluble in cold water and from sugars by being precipitated by alcohol. They have a high dextrorotation. Some idea as to the degree of hydrolysis which the starch has undergone can be secured from a determination of the gold number of the resulting dextrin. The manufacture of dextrin is usually carried out by autoclaving starch in the presence of a weak acid, *e.g.*, 5 to 15 pounds pressure with 1 per cent citric acid. Dextrins are largely used as adhesives. The "glue" of the postage stamps, envelopes, etc., is dextrin.

Various polymers of glucose have been prepared either directly from glucose or from starch, cellulose, or certain of the natural glycosides. When starch is fused *in vacuo* or dry-distilled,[21] it is converted into β-glucosan where the original 1-5 oxide ring of glucose is retained and where a new 1-6-oxide ring is added. Accordingly β-glucosan appears to be the 1-6-anhydride of β-glucose. An α-glucosan can be



β-Glucosan

prepared [22] by heating α-glucose at 150° C. *in vacuo*. It possesses the normal pyranose ring of glucose, but the dehydration takes place between the hydroxyl groups on carbon-1 and carbon-2. When



α-Glucosan

methylated it yields a 3-4-6-trimethyl-α-glucosan.

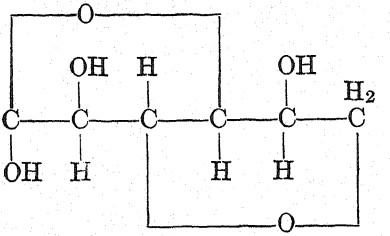
α-Glucosan can be polymerized by heating to give diglucosans, triglucosans, and tetraglucosans, the product obtained depending upon the temperature, pressure, and length of heating. Zinc chloride facilitates the polymerization. Polymeric products more or less analogous to these higher glucosans have been prepared from galactose, fructose, rhamnose, and arabinose.

A 3-6-anhydroglucose can be prepared by the action of hydrobromic acid on glucose pentaacetate, followed by heating with barium

[21] Irvine, J. C., and Oldham, J. W. H., Polymerisation of β-Glucosan. The Constitution of Synthetic Dextrins, *J. Chem. Soc.*, 127: 2903–2922 (1925); *cf.* also Pictet, A., Sur la transformation de la lévoglucosane en dextrine, *Helv. Chim. Acta*, 1: 226–230 (1918); Sur la polymérisation de la glucosane, *ibid.*, 4: 788–795 (1921).

[22] Pictet, A., and Ross., J. H., De l'influence de la pression sur la polymérisation de la lévoglucosane, *Helv. Chim. Acta.*, 5: 876–883 (1922); *cf.* also Cramer, M., and Cox, E. H., Sur la constitution de la glucosane, *ibid.*, 5: 884–887 (1922).

hydroxide and the cautious hydrolysis of this product with acids. 3-6-Anhydroglucose apparently has the structure:



3-6-Anhydroglucose

It is a glucofuranose derivative, has reducing properties, and forms both a phenylhydrazone and a phenylosazone.

Other anhydroglucoses have been prepared. 2-4-Anhydroglucose was obtained by the action of concentrated hydrochloric acid on β-glucosan, and 2-5-anhydroglucose or chitose results from the action of nitrous acid on glucosamine.

Glycogen or "Animal Starch."—Glycogen is the reserve carbohydrate of the muscles and especially of the liver. It is not found in green plants but is present in certain fungi, especially in yeast, as a reserve foodstuff. It is a white, amorphous powder, soluble in cold water to an opalescent, colloidal solution which gives a red-brown color with iodine, is precipitated by alcohol, does not reduce Fehling's solution, and is hydrolyzed to glucose.

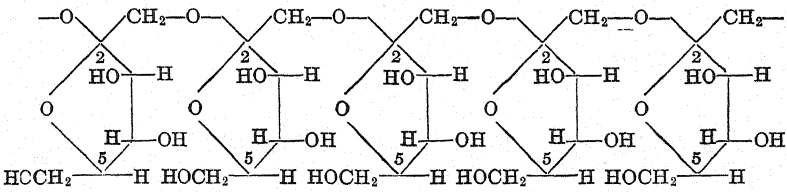
The structure of glycogen is apparently identical with that of β-amylose. Haworth,[23] from a study of the ratio of tetramethylglucose to trimethylglucose, concludes that the only difference between glycogen and starch is a shorter average minimum chain length. He found for glycogen that there were probably not more than 12 glucose residues in the chain, corresponding to a molecular weight of approximately 2,000. Glycogen does not show the phenomenon of retrogression to a more insoluble complex. A galactose polymer, apparently analogous to glycogen but designated as galactogen, occurs in the muscles of the snail, *Helix* sp.

Levulosans, Including Inulin and Inulides.—These are hydrolyzable to fructose. Inulin is a white, more or less crystalline powder, readily soluble (colloidal) in hot water, but slightly soluble in cold water (it can be recrystallized by this process), does not gelatinize on heating, does not turn blue with iodine, is not hydrolyzed by amylase or ptya-

[23] Haworth, W. N., and Percival, E. G. V., Polysaccharides VIII. Evidence of Continuous Chains of α-Glucopyranose Units in Starch and Glycogen, *J. Chem. Soc.*, 1342–1349 (1931); XI. Molecular Structure of Glycogen, *ibid.*, 2277–2282 (1932).

lin, but is hydrolyzed by inulase. Dahlia tubers contain 10 to 12 per cent of inulin.

The structure of inulin [24] appears to be that of a series of γ-fructose units linked together in glycosidal linkages.



On hydrolysis of methylated inulin the chief product is 3-4-6-trimethylfructofuranose with some 1-3-4-6-tetramethylfructofuranose. From the ratio of the tetramethyl to the trimethyl derivative, Haworth concludes that the chain length is in the neighborhood of 30 fructofuranose units with a molecular weight of approximately 5,000.

Other workers consistently find small amounts of glucose in highly purified inulin, and they regard the glucose as an integral part of the inulin molecule. The amount of glucose reported ranges from 1.5 per cent [25] to 3.7 per cent.[26] The latter value would account for a chain of 26 fructose units to 1 glucose unit, but the former value would necessitate a chain length of about 70 units and approximately doubling Haworth's molecular weight. Armstrong suggests that the glucose may be linked in inulin in a sucrose linkage.

That there are intermediate products between inulin and starch appears rather evident from the inulin literature, particularly in the case of the "inulides," and in the preparation of crystalline inulin, gummy, more or less pentosan-like substances are often removed in the purification process. These are poorly characterized, but on hydrolysis yield both fructose and glucose.

Inulin is apparently not utilized in the animal body, but when fed is excreted unchanged in the feces. In a depancreatized dog the feeding of inulin does not cause an increase in carbohydrate excretion, whereas fructose and galactose under similar conditions are quantitatively excreted as glucose.[27]

[24] Haworth, W. N., and Learner, A., Polysaccharides. I. The Structure of Inulin, *J. Chem. Soc.*, 619–625 (1928); Haworth, W. N., Hirst, E. L., and Percival, E. G. V., XV. The Molecular Structure of Inulin, *ibid.*, 2384–2388 (1932).

[25] Ohlmeyer, P., and Pringsheim, H., Über Inulin und die Inulinase, *Ber.*, 66: 1292–1295 (1933).

[26] Jackson, R. F., and McDonald, Emma, The Constant Occurrence of Nonreducing Disaccharides in Hydrolyzed Inulin, *Bur. Standards J. Research*, 5: 1151–1160 (1930).

[27] Bollman, J. L., The Utilization of Various Carbohydrates by the Depancreatized Animal, *Proc. Staff Meetings, Mayo Clinic*, 8: 67–68 (1933).

Mannosans.—The straw of cereals, the leaves and, to some extent, the wood of trees, and a part of the roots of certain plants are the usual sources of mannosans. Mannosans are apparently a structural component of plants. The mannosans closely resemble cellulose in many of their properties, except that upon acid hydrolysis they yield mannose rather than glucose.

In the mannan of the ivory nut the structure [28] is that of a series of 2-3-5-trihydroxymannose units with terminal linkages of 2-3-4-6-tetrahydroxymannose units. From a ratio of the trimethyl to tetramethyl hydrolysis products, mannan-A of the ivory nut appears to be a polysaccharide chain consisting of 71–76 mannopyranose residues linked through positions 1 and 4. It is accordingly analogous in structure to cellulose excepting that mannose replaces glucose.

Haworth *et al.*[29] report an unusual mannan which is called *mannocarolose* and which is produced when *Penicillium charlesii* is grown on culture media containing glucose. When methylated it gives rise to 2-3-4-6-tetramethylmannopyranose, 2-3-4-trimethylmannopyranose, and 2-3-dimethylmannopyranose. In this instance the mannopyranose residues are linked through positions 1 and 6, as are the glucose residues in gentiobiose. The chain length appears to be only approximately 9 pyranose units.

We have already referred to the alginic acid found in the brown algae as a polymer of mannuronic acid and the fact that mannitol occurs in association with the alginic acid. Bird and Haas [30] were unable to find any glucose derivatives in the brown algae which they investigated, and they suggest that the brown algae have a metabolism on a mannose basis rather than on a glucose basis. They state that the alginic acid occurs in the free state in the cell walls.

Galactosans.—These resemble mannosans, except that upon hydrolysis they yield galactose. They are widely distributed in nature as a structural element of plants (cell walls, etc.). So far as is known, there is no specific enzyme by which their hydrolysis can be accomplished. Galacturonic acid polymers occur in pectin and will be discussed under that heading.

Mucilages.—Some of the better-known mucilages are carrageen or "Irish moss," and agar-agar. They are the structural components of the cells of algae, and are prepared from "seaweeds." They are odorless, tasteless, and swell strongly in water but do not dissolve. When

[28] Klages, F., Zur Kenntnis der Steinnuss-mannane. I. Die Konstitution von Mannan A, *Ann.*, 509: 159–181 (1934).

[29] Haworth, W. N., Raistrick, H., and Stacey, M., Polysaccharides Synthesized by Micro-organisms. I. The Molecular Structure of Mannocarolose Produced from Glucose by *Penicillium charlesii* G. Smith, *Biochem. J.*, 29: 612–621 (1935).

[30] Bird, Gladys M., and Haas, Paul, On the Nature of the Cell Wall Constituents of *Laminaria* spp. Mannuronic Acid, *Biochem. J.*, 25: 403–411 (1931).

heated with water, they form sols which upon cooling set to a rigid gel. Such gels do not "melt" as readily as gelatin gels. A 1 per cent agar gel is fairly rigid. These gels are not liquefied by organisms which digest gelatin; consequently they are very useful in bacteriological laboratories. The animal body does not possess enzymes capable of digesting agar; consequently it cannot be utilized as a food and is sometimes prescribed to furnish bulk in cases of constipation. The presence of agar in the food can be readily determined by finding diatom shells in the ash, for the agar always contains diatom skeletons.

Agar, carrageen, and fucoidin are all sulfuric acid esters, where the ester group is a complex polysaccharide. The gelatinization of agar is the gelatinization of a salt of an agar sulfuric acid (agar—SO_3H). This salt may be metallic, as sodium, potassium, magnesium, calcium, etc., or it may be an organic base, such as aniline or an alkaloid, and satisfactory gels will be formed. The "agar acid" itself will not gelate, and is so strongly acid that it undergoes autohydrolysis upon heating. A 1 per cent solution of the free agar acid has a *p*H of approximately 2.0.[31]

On acid hydrolysis agar-agar yields galactose, or a carbohydrate isomeric with galactose. Besides 20 to 28 per cent of galactan, there is evidence for the presence of mannans and fucosans. The question of the exact composition of the carbohydrate portion of agar or carrageen is still more or less uncertain, although these two mucilages have been worked with more than any others.

GUMS.—Most of the gums are not represented by the formula $(C_6H_{10}O_5)_x$, but are in reality more or less glycoside-like compounds consisting of hexoses or pentoses (or both), combined with other substances, generally complex acids. On hydrolysis they usually yield galactose, arabinose, or xylose, either alone or in mixtures. Certain gums have yielded an acid which apparently has the formula $(C_{23}H_{38}O_{22})_x$. Gum acacia or gum arabic is probably the best-known example of this group of compounds.

Arabic acid is a relatively strong acid,[32] a 1 per cent solution showing a *p*H value of 2.70, which is equivalent to a 0.02*N* solution of hydrochloric acid. It is, however, not an ester of either sulfuric acid or of phosphoric acid, since it contains no sulfur, phosphorus, or chlorine. From its titration values with sodium and barium hydroxides, the equivalent weight of the acid appears to be approximately 1,200.

[31] Hoffman, W. F., and Gortner, R. A., The Electrodialysis of Agar. A Method for the Preparation of the Free Agar-Acid, *J. Biol. Chem.*, 55: 371–379 (1925); *cf.* also Haas, P., and Hill, T. G., on Carrageen, *Chondrus Crispus*, *Ann. Applied Biol.*, 7: 352–362 (1921); II. On the Occurrence of Ethereal Sulphates in the Plant, *Biochem. J.*, 15: 469–476 (1921).

[32] Thomas, A. W., and Murray, H. A., Jr., A Physico-Chemical Study of Gum Arabic, *J. Phys. Chem.*, 32: 676–697 (1928).

Gum arabic, therefore, can be looked upon as the calcium or calcium-magnesium salt of arabic acid. Among the decomposition products of arabic acid there has been isolated an aldobionic acid which is a 6-glucuronosido-galactopyranose.[33]

IMMUNOLOGICALLY SPECIFIC POLYSACCHARIDES.—Various workers had noted that certain bacterial cultures contain a "soluble specific substance" which reacts with antisera to form precipitates. Thus, the fluid cultures of pneumococci contain a substance which is a specific precipitant for anti-pneumococcus serum even when a dilution of the soluble specific substance is as high as 1 : 6,000,000.

It remained for Heidelberger, Avery, and Goebel [34] to discover that the soluble material which is present in the bacterial cultures was of a carbohydrate nature. Apparently this carbohydrate is a hapten (*cf.* Chapter XX) and a specific polysaccharide elaborated by the particular microorganism. In the initial study of the nature of these specific polysaccharides, Heidelberger and Goebel [35] made a careful investigation of the polysaccharide of Type III Pneumococcus. The yield of this polysaccharide is about 2 grams for each 10 liters of glucose broth culture medium. The polysaccharide which they isolated is colloidal, strongly acidic, possesses an apparent micellar weight lying somewhere between 1,000 and 5,600, and is built up largely of an aldobionic acid having the formula $C_{11}H_{19}O_{10} \cdot COOH$, and consisting of 1 molecule of glucose and 1 molecule of glucuronic acid joined in a glycosidal union, probably through carbon-1 of the glucuronic acid to carbon-6 of the glucose residue.

After the initial discovery of the immunologically specific polysaccharides, a great many workers [36] became active in this field. Specific polysaccharides have been isolated from tubercle bacilli, streptococci, gonococci, meningococci, staphylococci, members of the phytomonas

[33] Challinor, S. W., Haworth, W. N., and Hirst, E. L., The Compound Uronic Acids. Structure of the Aldobionic Acid from Gum Arabic, *J. Chem. Soc.*, 258–265 (1931); *cf.* also Hotchkiss, R. D., and Goebel, W. F., The Synthesis of the Aldobionic Acid of Gum Acacia, *J. Am. Chem. Soc.*, 58: 858–859 (1936).

[34] Heidelberger, M., and Avery, O. T., The Soluble Specific Substance of Pneumococcus, *J. Exptl. Med.*, 38: 73–79 (1923); II, *ibid.*, 40: 301–316 (1924); (and Goebel, W. F.), III. *ibid.*, 42: 727–745 (1925).

[35] Heidelberger, M., and Goebel, W. F., The Soluble Specific Substance of Pneumococcus. IV. On the Nature of the Specific Polysaccharide of Type III Pneumococcus, *J. Biol. Chem.*, 70: 613–624 (1926); V. On the Chemical Nature of the Aldobionic Acid from the Specific Polysaccharide of Type III Pneumococcus, *ibid.*, 74: 613–618 (1927); Heidelberger, M., Immunologically Specific Polysaccharides, *Chem. Rev.*, 3: 403–423 (1927); Heidelberger, M., and Kendall, F. E., Some Physicochemical Properties of Specific Polysaccharides, *J. Biol. Chem.*, 95: 127–142 (1932); The Molecular Weight of Specific Polysaccharides, *ibid.*, 96: 541–558 (1932).

[36] For literature see Landsteiner, K., The Specificity of Serological Reactions, Chapter VI, Charles C. Thomas, Baltimore, Maryland (1936).

and pasteurella groups, *B. influenzae*, *B. lactis aerogenes*, Salmonella bacilli, *B. dysenteriae*, bacteria of the brucella group, *B. proteus*, *B. anthracis*, Spirochaetes, yeasts, and Rickettsiae, and presumably are present in many other organisms which have not yet been investigated. Similar compounds have even been reported to be present in fungi.

All the earlier work on the chemistry of the immunologically specific polysaccharides apparently needs to be reviewed in the light of the observations [37] that the earlier preparations probably represented decomposition products and that the compounds which are actually present and which show maximum serological activity are more or less readily destroyed by treatment with alkali. This observation has led to some revision with regard to the constituents present in the specific polysaccharides of Types I, II, and III Pneumococci. The polysaccharide from Type I Pneumococcus contains nitrogen, acetyl, and uronic acid groups. Assuming a molecular weight of 600, the polysaccharide contains two atoms of nitrogen, one acetyl group, and two galacturonic acid residues. One of the nitrogen atoms is free as amino nitrogen. The carboxyl groups of the uronic acid residues are free. Tentatively the fundamental unit is a trisaccharide containing two galacturonic acid residues and an unidentified residue containing two nitrogen atoms. Pneumococcus Type IV contains an amino sugar and acetyl residues. Uronic acids appear to be absent. The polysaccharide appears to be more nearly related to a chitin residue.

Nitrogen seems to be absent from the polysaccharides from Types II and III Pneumococci. Here the polysaccharide appears to be an aldobionic acid, possibly identical with the glucoglucuronic acid which was earlier isolated. Aldobionic acids are not limited to these bacterial gums but are also present in such gums as gum acacia.[38] A galactoglucuronic acid was isolated from gum acacia in a 28 per cent yield.

A specific bacterial enzyme has been reported [39] which acts on the specific polysaccharide of Type III Pneumococcus. This enzyme, when injected into mice inoculated with the Type III organism, showed distinct prophylactic and curative effects which were specific for Type III Pneumococcus.

CHITIN.—Chitin or polymerized acetylglucosamine bears essentially the same relationship to glucosamine that cellulose does to glucose. It is the structural element of the skeletons of the *Arthropoda*. In the animal kingdom, with but few exceptions, it is limited to the invertebrates. It forms the exoskeletons of insects, crustaceans, etc.,

[37] Heidelberger, M., Kendall, F. E., and Scherp, H. W., The Specific Polysaccharides of Types I, II, and III Pneumococcus, *J. Exptl. Med.*, 64: 559–572 (1936).

[38] Butler, C. L., and Cretcher, L. H., The Composition of Gum Arabic, *J. Am. Chem. Soc.*, 51: 1519–1525 (1929).

[39] Avery, O. T., and Dubois, R., The Specific Action of a Bacterial Enzyme on Pneumococci of Type III, *Science*, 72: 151–152 (1930).

and in addition is one of the major constituents in the lenses of the eyes, tendons, and the lining of the respiratory, excretory, and digestive tracts. It is to invertebrates what cellulose is to plants.

Chitobiose is the hydrolysis product of chitin and differs from cellobiose only in that the —OH group on carbon-2 is replaced by an acetylamino group. X-ray diffraction studies [40] indicate that chitin is beautifully crystalline, containing a unit cell $9.25 \times 10.46 \times 19.25$ Å. In this crystal cell the alternate parallel chains of glucosamine units are oriented in a reverse direction, four such parallel chains making up the unit cell as shown in Fig. 151.

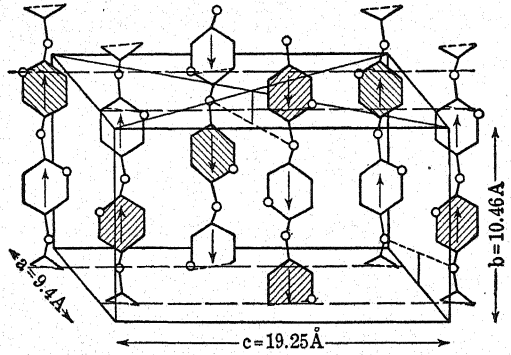
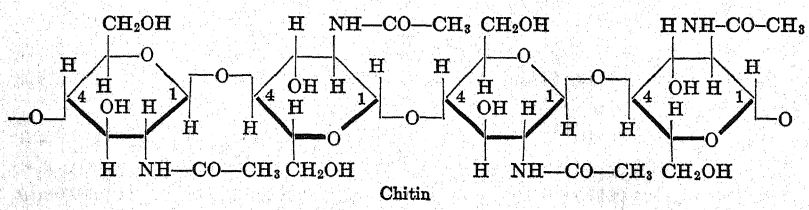


FIG. 151.—The arrangement of the glucosamine units in the unit cell of chitin as shown by X-ray data. (After Clark.)

Chitin is extremely resistant to bacterial action and to the usual process of decay. Wing remains of Coleoptera from the middle Eocene (±25,000,000 years ago) were found [41] to still contain chitin as proved by the isolation of chitosamine hydrochloride.

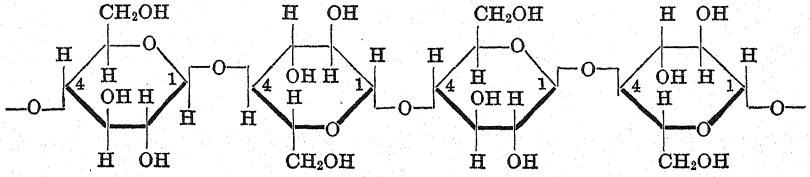


CELLULOSE.—The term cellulose covers a class of compounds found in the woody portion of plants. These compounds are characterized by possessing the formula $(C_6H_{10}O_5)_x$, by being insoluble in water

[40] Clark, G. L., and Smith, A. F., X-Ray Diffraction Studies of Chitin, Chitosan, and Derivatives, *J. Phys. Chem.*, 40: 863–879 (1936).

[41] Abderhalden, E., and Heyns, K., Nachweis von Chitin in Flügelresten von Coleopteren des oberen Mitteleocäns (Fundstelle Geiseltal), *Biochem. Z.*, 259: 320–321 (1933).

and in all organic solvents, soluble in "Schweitzer's reagent" (ammoniacal copper hydroxide[42]), soluble in zinc chloride dissolved in twice its weight of hydrochloric acid, soluble in molten $FeCl_3 \cdot 6H_2O$, in which solution it is more or less completely hydrolyzed to glucose.



Cellulose

The literature dealing with cellulose is very voluminous, so that only a casual reference can be made to some of the outstanding characteristics of this compound. Cellulose is relatively inert toward mild chemical reagents. Treated with dilute alkalies there is little immediate reaction. Stronger alkalies, such as 13 per cent sodium hydroxide, form "hydrocellulose" and "mercerize" the fiber. Dilute sulfuric acid forms a "hydrocellulose," while stronger sulfuric acid converts the cellulose fibers into "vegetable parchment." Dilute nitric acid forms "oxycellulose," and concentrated nitric acid forms nitrocellulose.

Irvine and Soutar[43] hydrolyzed cellulose with acid, and on the theory that cellulose is composed wholly of glucose, obtained 85 per cent of the theoretical yield of glucose as the crystalline sugar. They believe that glucose is the only product formed by the hydrolysis of cellulose. Upon destructive distillation, cellulose yields acetone, acetic acid, and methyl alcohol. The highest nitrate is three NO_2 groups to each six carbon atoms; the highest acetate is three acetyl groups to each six carbon atoms, indicating that only three hydroxyl groups in each molecule of glucose remain uncombined.

We have already noted that the starch molecule consists of regular chains of α-glucopyranose units joined in a glucosidal linkage at carbon-4. The cellulose molecule differs from the starch molecule only in that it is a regular chain of β-glucose residues joined in glucosidal linkage at carbon-4. By comparing the formula for starch with cellulose, it will be noted that in the starch formula all the oxygens of the pyranose rings are on one side of the molecular chain, whereas in cellulose there is an alternation of the oxygens in the pyranose rings.

X-ray studies[44] have shown that cellulose is crystalline or at least

[42] Neale, S. M., The Nature of Solutions of Cellulose in Cuprammonium Hydroxide, *J. Text. Inst.*, 16: 363–369 (1925).

[43] Irvine, J. C., and Soutar, C. W., The Constitution of Polysaccharides. Pt. II. The Conversion of Cellulose into Glucose, *J. Chem. Soc.*, 117: 1489–1500 (1920).

[44] Clark, G. L., Cellulose as It Is Completely Revealed by X-Rays, *Ind. Eng. Chem.*, 22: 474–487 (1930).

gives an X-ray diffraction pattern. The unit cell is $7.9 \times 8.3 \times 10.3$ Å and contains three cellobiose units in the arrangement shown in Fig. 152. Four cellobiose units are situated at the edges of the parallelogram and accordingly are shared by other unit cells so that only one-half of each of these four units is counted in the unit cell. One cellobiose unit is situated in the center of the unit cell. In Fig. 152 only the pyranose rings and the oxygens linking the glucose units together have been depicted.

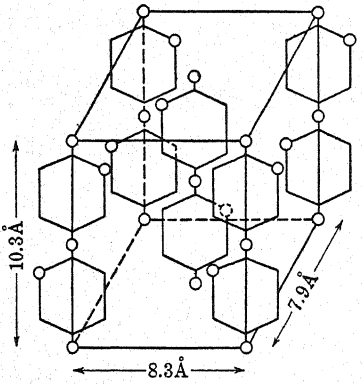


FIG. 152.—The arrangement of the glucose units in the unit cell of cellulose as shown by X-ray data. (After Clark.)

Using the ratio of trimethylglucose to tetramethylglucose, the chain length of cellulose was found [45] to correspond to from 100 to 200 glucose units. This would mean a minimal micellar weight of from 16,000–32,000 and a molecular length of from 500 to 1,000 Å. Osmotic-pressure measurements, diffusion experiments, and the studies with the ultracentrifuge are all in fair agreement that there are in the neighborhood of 200 glucose units in the chain. These chains of cellulose units are then arranged essentially parallel to each other in a space relationship similar to that shown in Fig. 152. Probably the factors which tend to hold these molecular chains at a fixed distance from each other reside in covalence forces [46] radiating from the oxygen groups, both the oxygen of the pyranose ring and the oxygens of the hydroxyl groups being involved. Figures 153 and 154 illustrate these covalence forces.

Cellulose fibers, in general, show little or no swelling in a longitudinal direction, probably because the linkages in the longitudinal direction of the fiber are the linkages of primary valence. On the other hand, cellulose fibers swell greatly in a lateral direction. Here covalence forces are involved. The water molecules of the medium are adsorbed upon the oxygen atoms, which tends to increase the distance between the parallel chains of glucose molecules.

Sponsler and Dore [47] believe that three changes take place in the

[45] Haworth, W. N., and Machemer, H., Polysaccharides. X. Molecular Structure of Cellulose, *J. Chem. Soc.*, 2270–2277 (1932).

[46] Sponsler, O. L., and Dore, W. H., The Structure of Ramie Cellulose as Derived from X-Ray Data, Colloid Symposium Monograph, Vol. 4, pp. 174–202, Chemical Catalog Company, New York (1926).

[47] Sponsler, O. L., and Dore, W. H., The Structure of Mercerized Cellulose. I. The Space Lattice of Mercerized Ramie Cellulose as Developed from X-Ray Data, *J. Am. Chem. Soc.*, 50: 1940–1950 (1928).

space lattice structure of ramie cellulose that has been "mercerized" by treatment with sodium hydroxide: (1) a shift in the position of the chain of glucose residues with respect to the neighboring chains,

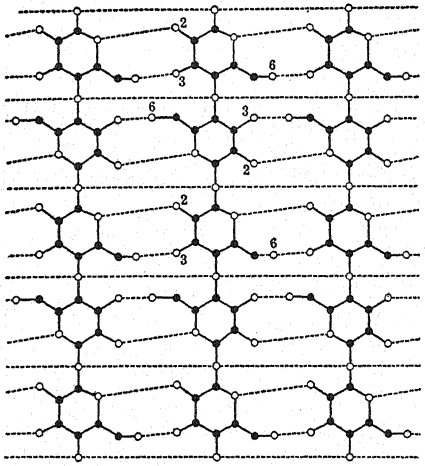


FIG. 153.—Structure of cellulose. Tangential section through a ramie fiber showing three chains of glucose units. Dark lines indicate primary valence bonds. Broken lines indicate probable general direction of secondary valence forces. (After Sponsler and Dore.)

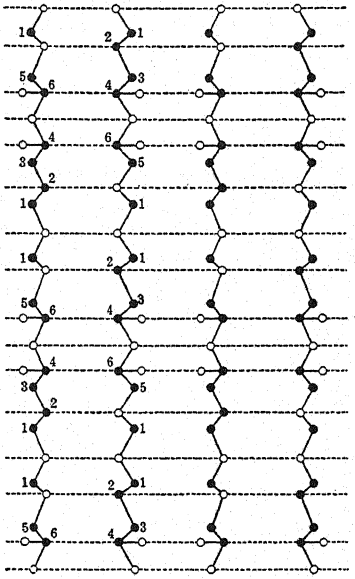


FIG. 154.—Structure of cellulose. A radial section through a ramie fiber showing primary and secondary valence bonds. (After Sponsler and Dore.)

(2) a partial rotation of the alternate glucose units in each chain, and (3) a shift in the spatial relationships of the hydroxyl group which is attached to the sixth carbon atom.

They find that no mercerization takes place in alkali less concentrated than 13 per cent and state that it is extremely unlikely that the mercerization process involves either the migration of an oxygen bridge, enolization, or a molecular rearrangement of the glucose units.

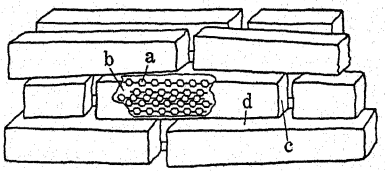


FIG. 155.—A suggested arrangement of the cellulose micelles in the cell-wall structure. (After Seifriz.)

Seifriz [48] suggests that in the cell walls of plants the cellulose micelles are built into a cellulose structure which he showed diagrammatically in a figure which is reproduced as Fig. 155.

[48] Seifriz, W., The Origin, Composition, and Structure of Cellulose in the Living Plant, *Protoplasma*, 21: 129–159 (1934).

When cellulose is dispersed in cellulose solvents, the orientation of the cellulose micelles, which is so characteristic of natural fibers, is destroyed. If the cellulose is regenerated without orientation, the threads or sheets which result have a much lower tensile strength than when the micelles are reoriented in the regeneration process. In the flax fiber the cellulose micelles have a very high degree of orientation, and flax fiber has a tensile strength comparable to that of the best steel. In a poorly oriented viscose or acetate rayon fiber the tensile strength may be less than 20 per cent that of a flax fiber.

It is usually assumed that the cotton fiber is practically pure cellulose. However, in a series of recent papers Mrs. Farr [49] concludes that the true "cellulose" is a tiny microscopic particle approximately uniform in size ($1.0\,\mu \times 1.4\,\mu$) and near the limit of microscopic visibility. Such particles were visibly ellipsoidal in shape and doubly refracting. She believes that these particles are imbedded in the ordinary fiber in a pectin-like matrix and that it is these tiny "crystalline" particles which are responsible for the X-ray pattern. Pectic solvents separate the "cellulose" from the pectin matrix, and she states that the viscosities of cuprammonium solutions of cellulose are largely the viscosities of the pectin matrix material and that the crystallites of cellulose are not dispersed in the cuprammonium solution. By diluting such solutions the crystallites will sediment and may be separated from the pectin matrix.

The photomicrographs which Mrs. Farr has obtained, particularly those showing the development of the cotton fiber from its earliest stages to the final stages, support her contention most admirably. If she is correct in her contentions, and if pectin materials are present in cellulose fibers, we will have to discard a great many of our present beliefs.

[49] Farr, W. K., and Clark, G. L., Cotton Fibers. II. Structural Features of the Wall Suggested by X-ray Diffraction Analyses and Observations in Ordinary and Plane-Polarized Light, *Contrib. Boyce Thompson Inst.*, 4: 273–295 (1932); Farr, W. K., and Eckerson, S. H., Formation of Cellulose Membranes by Microscopic Particles of Uniform Size in Linear Arrangement, *ibid.*, 6: 189–203 (1934); Separation of Cellulose Particles in Membranes of Cotton Fibers by Treatment with Hydrochloric Acid, *ibid.*, 6: 309–313 (1934); Farr, W. K., and Sisson, W. A., X-Ray Diffraction Patterns of Cellulose Particles and Interpretations of Cellulose Diffraction Data, *ibid.*, 6:315–321 (1934).

CHAPTER XXVII

THE GLYCOSIDES AND SAPONINS

The synthetic and the naturally occurring glycosides are similar in structure, and the formula may be represented by sugar —O—R, where R represents the foreign (non-sugar) group attached to carbon-1 of the sugar. This may be coniferyl alcohol as in the glucoside *coniferin*, or salicyl alcohol as in *salicin*, or aniline, or benzaldehyde, etc., as in various glycosides.

The glycosides are widely distributed in nature but usually occur in small amounts. The non-sugar residue is in most instances an aromatic compound. Only sugars capable of forming a δ-oxide ring form glycosides in nature, and most of the naturally occurring glycosides are compounds of glucose, although arabinose, xylose, ribose, rhamnose, galactose, mannose, and fructose have been isolated from glycosides.

The α- and β-forms of the glucosides have already been discussed. The methyl and ethyl glucosides and galactosides and the methyl xylosides are known in both α- and β-forms, but *all naturally occurring glucosides, so far as is known, occur only in the β-form* and are hydrolyzed only by emulsin.

The rotatory power of an unknown glucoside (*e.g.*, the α-form) can be calculated [1] from the optical rotation of the known β-form because

$$\text{Rotation of } \frac{\alpha + \beta \text{ glucosides}}{2} = \text{rotation of } \frac{\alpha + \beta \text{ glucose}}{2}$$

when R is not optically active.

The role of the glycosides in the plants has been interpreted as a mechanism whereby the substances which have great physiological activity are held inert until they are needed in the metabolism of the plant or in rendering poisonous substances inert so that they will not injure the plant cells.[2, 3, 4] Glycosides are most commonly found in

[1] Hudson, C. S., The Significance of Certain Numerical Relations in the Sugar Group, *J. Am. Chem. Soc.*, 31: 66–86 (1909).

[2] Combes, R., Recherches biochimiques expérimentales sur le rôle physiologique des glucosides chez les végétaux, *Rev. gén. botan.*, Vols. 29 and 30 (1917–18). This article, including a total of 224 pages begins on p. 321 of Vol. 29 and is continued in 13 instalments, ending on p. 363 of Vol. 30.

[3] Combes, R., Immunité des végétaux vis-à-vis des principes immédiats qu'ils élaborent, *Compt. rend.*, 167: 275–278 (1918).

[4] Clark, E. D., Note on the Blackening of the Leaves of the Wild Indigo (*Baptisia tinctoria*) and the Isolation of a New Phenol, Baptisol, *J. Biol. Chem.*, 21: 645–660 (1915).

the fruit, bark, or roots of plants, although they frequently occur in the leaves. They are usually levorotatory, crystalline, colorless, bitter, soluble in water or in alcohol. In order to isolate them without hydrolysis, it is desirable to destroy their accompanying enzyme by heat. They are easily hydrolyzed by emulsin or by dilute mineral acids.

The glycosides may be classified according to the nature of the non-sugar part of the molecule, under phenols, alcohols, aldehydes, acids,

TABLE LXXIV

CERTAIN NATURALLY OCCURRING GLYCOSIDES

Name	Hydrolytic Products	Source
	I. PHENOLS	
Arbutin	Glucose + hydroquinone	*Arbutus uva ursi*
Phlorizin	Glucose + phloretin	Bark of *Rosaceae*
	II. ALCOHOLS	
Coniferin	Glucose + coniferyl alcohol	Bark of fir tree
Populin	Glucose + saligenin + benzoic acid	Bark of *Populus*
Salicin	Glucose + saligenin	Bark of willow (*Salix* sp.)
	III. ALDEHYDES	
Amygdalin	2 Glucose + *d*-mandelonitrile	Seeds of bitter almond
Dhurrin	Glucose + *p*-oxymandelonitrile	Leaves of *Sorghum vulgare*
Linamarin	Glucose + acetonecyanhydrin	Leaves of young flax plants and flax seed
Prulaurasin	Glucose + racemic mandelonitrile	Leaves of *Prunus laurocerasus*
Prunasin	Glucose + *d*-mandelonitrile	Young twigs of *Prunus padus*
Sambunigrin	Glucose + *l*-mandelonitrile	Leaves of common elder (*Sambucus niger*)
	IV. ACIDS	
Gaultherin	Glucose + methylsalicylate	Bark of *Betula lenta* or *Gaultheria procumbens*
Jalapin	Glucose + jalapinolic acid	Roots of *Jalapa orizabenzis*
	V. OXYCUMARIN DERIVATIVES	
Aesculin	Glucose + aesculetin	Bark of horse-chestnut (*Aesculus hippocastanum*)
Fraxin	Glucose + fraxetin	Bark of the ash (*Fraxinus* sp.)
	VI. OXYANTHRAQUINONE DERIVATIVES	
Ruberythric acid	Glucose + alizarin	Madder, the roots of *Rubia tinctorum*

TABLE LXXIV—*Continued*

Name	Hydrolytic Products	Source
	VII. Oxyflavone Derivatives	
Apiin	Apiose + apigenin	Leaves of parsley, celery, etc.
Quercitrin	Rhamnose + quercetin	Bark of oak
Rutin	Glucose + rhamnose + quercetin	Petals of the violet, leaves of *Ruta graveolens*, etc.
Xanthorhamnin	2 Rhamnose + galactose + rhamnetin	Fruits of various species of *Rhamnus*
	VIII. Mustard Oils	
Sinigrin	Glucose + allyl isothiocyanate + $KHSO_4$	Black mustard seeds
	IX. Anthocyans	
Cyanin	2 Glucose + cyanidin	Flowers of cornflower, rose, etc.
Delphinin	2 Glucose + 2 *p*-oxybenzoic acid + delphinidin	Flowers of larkspur
Oenin	Glucose + oenidin	Skins of purple grapes
Pelargonin	2 Glucose + pelargonidin	Flowers of geranium
	X. Digitalis Group	
Digilanide-A	3 Digitoxose ($C_6H_{12}O_4$) + glucose + acetic acid + digitoxigenin	Leaves of foxglove (*Digitalis purpurea*)
Digilanide-B	3 Digitoxose + glucose + acetic acid + gitoxigenin	Leaves of foxglove
Digilanide-C	3 Digitoxose + glucose + acetic acid + digoxigenin	Leaves of foxglove
	XI. Sapogenins	
Digitonin	4 Galactose + xylose + digitogenin ($C_{27}H_{44}O_5$)	Leaves of foxglove
Gitonin	3 Galactose + pentose + gitogenin ($C_{27}H_{44}O_4$)	Leaves of foxglove
Sarsasaponin	3 Glucose + sarsasapogenin ($C_{27}H_{44}O_3$)	*Radix sarsaparillae*
	XII. Various	
Dibenzoylglucoxylose	Glucoxylose + benzoic acid	Leaves of *Daviesia latifolia*
Indican	Glucose + indoxyl	Leaves of *Indigofera*

etc. A list of a few of the more important of the naturally occurring glycosides is shown in Table LXXIV.

Certain glycosides contain two hexose molecules, *e.g.*, amygdalin which contains 2 glucose + benzaldehyde + HCN (as the nitrile). By appropriate technic one carbohydrate group can in some instances be hydrolyzed off, yielding a new glycoside containing only *one* hexose.

Both on account of the very small amount of glycosides present in plant tissues and the fact that, as a rule, glycosides do not form characteristic insoluble derivatives which allow for their isolation and identification, it is difficult to discover new glycosides and still more difficult to determine their constitution. Certain biochemical methods have assisted materially in this work:

1. *Bourquelot's method* for examining a plant for glycosides consists in determining the reducing sugar and the optical rotation of an extract of the plant tissue. Then he adds emulsin and after a period of incubation redetermines the reducing sugars and the optical rotation. An increase in optical rotation indicates the presence of β-glycosides, and the amount of change may give a rough estimation of the amounts which are present.

2. The nature of the sugar can be more or less accurately determined, according to ter Meulen, by adding the different sugars to the glycoside + enzyme mixture. The sugar which retards the enzyme action is probably the one in the glycoside, inasmuch as the hydrolysis is an equilibrium reaction.

$$\text{Glycoside} + \text{enzyme} + H_2O \rightleftharpoons \text{enzyme} + \text{sugar} + \text{non-sugar residue}$$

This consideration possibly led to Bourquelot's *enzyme synthesis* work which established definitely that enzyme actions were equilibria and were reversible. The first demonstration of synthesis by enzyme action was carried out in the case of glycosides. Emulsin was added to a concentrated solution of the split products of the glycoside, and the mixture was incubated at 37° C. It was found that the equilibrium

$$\text{Glycoside} + H_2O \rightleftharpoons \text{sugar} + \text{non-sugar residue}$$

was shifted toward the left, *i.e., the amount of reducing sugar decreased.* The synthesis of glycosides was thus definitely proved. Upon dilution of the system, the reaction again shifted to the right, and hydrolysis of the glycoside took place.

Bridel and Béguin [5] used this method of enzyme synthesis for the preparation of ethyl-*l*-arabinose by adding emulsin to a solution of pure *l*-arabinose in alcohol and allowing the mixture to stand for 173 days, adding more emulsin on the twenty-seventh and forty-third days. They state that the compound isolated was α-ethyl-*l*-arabinoside and suggest that possibly the commercial emulsin contained as a specific enzyme, α-*l*-arabinosidase.

Cyanogenetic or Cyanophoric Glycosides.—These glycosides have received a considerable amount of attention not only because of their practical importance (they are present in such substances as

[5] Bridel, M., and Béguin, C., Synthèse biochimique, à l'aide de l'émulsine des amandes, de l'ethyl-*l*-arabinoside α, *Compt. rend.*, 182: 812–814 (1926).

bitter almonds, linseed cake, sorghum, and other fodder plants, etc.), but also because of theoretical interest as to their function in plants. They contain nitrogen as HCN, and because of that fact can be more or less easily estimated. HCN is fairly common among higher plants and occurs, in the majority of cases, combined in glycosides.

Although the cyanogenetic glycosides are somewhat rare as compared with saponins, they have a much wider distribution than was once supposed, and are present in many economic plants. Flax contains a small amount of HCN in the dry seed (0.008 per cent HCN), but as much as 0.135 per cent has been found in the germinated seedlings. The same increase occurs in the germination of bitter almonds. The stage of development at which the maximum amount is reached may be different for different plants. Thus, in flax the maximum occurs when the seedlings are 4 to 5 inches high, while in *Lotus arabicus* the maximum is at the time of flowering. In sorghum there appears to be no HCN in the seed, but Willaman found the maximum amount in seedlings about 3 inches high, decreasing to practically zero in the mature plant.

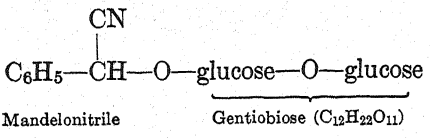
Uncombined HCN has been found in plants in a few instances, but it is extremely rare, and the presence of HCN may be considered as evidence of the presence of a cyanophoric glycoside. Therefore, the quantitative determination of the cyanophoric glycosides consists in the determination of HCN, formed by the hydrolysis of the glycosides, (*a*) by autolyzing the plant tissue, or (*b*) by addition of acid, or (*c*) by incubating, following the addition of emulsin, and distilling off the HCN into alkali. Autolysis was the first method employed, in fact the autolysis of crushed bitter almonds was the first means by which HCN was identified as a plant constituent.

A great deal of attention has been given to methods for the determination of HCN, and the various factors governing its quantitative liberation, quantitative distillation, and measurement have been exhaustively studied. There is still much room for improvement in the present methods. In most cases one deals with quantities ranging from a fraction of a milligram to one or two milligrams of HCN, and the measurement depends upon the conversion of ferrous salts to Prussian blue. The conditions of formation must be such that the pigment will remain suspended in a colloidal sol, so that the measurement can be made colorimetrically.

Amygdalin, $C_{20}H_{27}O_{11}N$, is probably the best known of the glycosides. It is obtained from the kernels of the bitter almond, *Prunus amygdalus*. It also occurs in smaller amounts in the kernels of the peach, plum, apricot, and most fruits belonging to the *Rosaceae*.

Liebig and Wöhler, in 1837, found that amygdalin was hydrolyzed to glucose, benzaldehyde, and HCN. Caldwell and Courtauld showed that the reaction took place in two stages when acids were used as the

hydrolyzing agents, one glucose radical being split off before the second one was attacked. The structure has been shown to be



Emulsin hydrolyzes the two glucose bonds at about the same rate, so that the end products of emulsin hydrolysis are two molecules of glucose and mandelonitrile which further hydrolyzes into benzaldehyde and HCN. Amygdalase hydrolyzes off only one glucose radical, forming a new glycoside, *prunasin*, which in turn is hydrolyzed by the enzyme *prunase*. The enzyme prunase was first found in the leaves of the cherry laurel, hence the name. "Emulsin" therefore contains two enzymes, amygdalase and prunase. Prunase cannot act until the amygdalase has split off one glucose radical. This is another example of enzyme biological specificity and indicates that one glucose radical shields the rest of the molecule from the enzyme.

Three glycosides of the same formula and composition as prunasin are known:

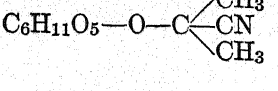
1. Prunasin — *d*-mandelonitrile-glucoside
2. Prulaurasin — *d-l*-(racemic)-mandelonitrile-glucoside
3. Sambunigrin — *l*-mandelonitrile-glucoside

This is one of the few instances where the *d*, *l*, and racemic forms of a compound all occur in nature.

Dhurrin occurs in *Sorghum vulgare* and was isolated by Dustan and Henry in 1902. Its structure is *p*-hydroxymandelonitrile-glucoside, hydrolyzing to glucose, HCN, and *p*-hydroxybenzaldehyde. It also occurs in the forage crop, "Sudan grass."

Vicianin, $C_{19}H_{25}O_{10}N$, occurs in the seeds of the wild vetch, *Vicia angustiafolia*, and yields on hydrolysis HCN, benzaldehyde, and a disaccharide, vicianose (glucose + arabinose). It may be noted that this glycoside is similar to amygdalin except that one glucose radical of amygdalin has been replaced by arabinose. The wild vetch seed contains a relatively small amount of glycoside (HCN = 0.0033 per cent of the seed), but a small amount may produce undesirable effects. The vetch is one of the so-called "non-separable" weed seeds in grain, and the presence of vetch in wheat will produce a pronounced benzaldehyde odor in bread doughs made from the flour.

Linamarin or *phaseolunatin*,



hydrolyzes to β-glucose, acetone, and HCN. It occurs in flaxseed, flax plants, etc.

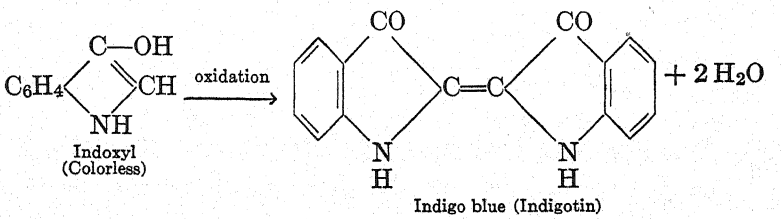
Phlorizin, yielding, as the products of acid hydrolysis, glucose, phloroglucinol, and *p*-oxyhydratropic acid,

$$[(p)OH—C_6H_4—CH(CH_3)—COOH],$$

is found in the bark of the apple, pear, and other rosaceous trees. Phlorizin possesses the remarkable property of causing "artificial diabetes" or glycosuria when taken internally, or better when injected subcutaneously (in oil). Much of the experimental work on diabetes has been suggested by experiments made on phlorizinized dogs.

Salicin, a glycoside hydrolyzing to β-glucose and saligenin (*o*-hydroxybenzyl alcohol) occurs in the bark of most species of *Salix* (willows). This glycoside is assuming increased importance because of the action of saligenin as a local anesthetic. Certain salicin derivatives show abnormal optical rotations.[6]

Indican, $C_{14}H_{17}O_6N$, on hydrolysis yields glucose and indoxyl. Indoxyl is the leuco base of indigo blue to which it passes on oxidation.



Indoxyl (Colorless)

Indigo blue (Indigotin)

The glycoside occurs in the indigo plants and can be extracted by acetone. A specific enzyme is present in the leaf which hydrolyzes the glycoside, and the indoxyl in the presence of air and of an *oxidase* is converted to the indigo blue. The sugar radical must be split off before oxidation can take place. This is one of the best-known cases where one enzyme (glucosidase) must act to produce a compound before a second enzyme (oxidase) can act to produce a third substance.

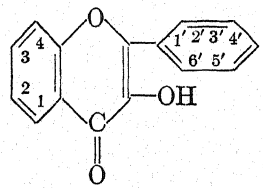
The production of indigo from indican was at one time one of the world's great industries, and the indigo planters of India were practically ruined by Baeyer's synthesis of indigo. In carrying out the successful synthesis, the Badische Company spent nearly $5,000,000 during seventeen years of research before a pound of synthetic indigo had been sold. At the present time natural indigo is not a serious competitor of synthetic indigo except in the orient, where cheap labor

[6] Kunz, A., Studies on Salicin. I. Exceptional Rotations of the Halogeno-tetra-acetyl Derivatives of Salicin. A New Synthesis of Salicin, *J. Am. Chem. Soc.*, 48: 262–268 (1926).

is available and an inferior product is acceptable. The crude natural indigo often contains only 5 to 7 per cent of dyestuff.

Although indigo occurs exclusively as a glycoside in the plant kingdom, and in the animal body as a decomposition product of tryptophane formed by bacterial action, a brom-substituted indigo occurs in a gland in the molluscs, *Murex brandaris* and *Murex trunculus*. This compound, 6.6′-dibrom indigo, was known to the ancients as "Tyrian purple." Friedländer [7] isolated 1.5 grams of the dyestuff from 12,000 molluscs. Research has shown that the synthetic 5.5′-dibrom indigo dyes cloth a clearer purple and is much to be preferred to the "Tyrian purple" as a dyestuff. 6.6′-Dibrom indigo is one of the few naturally occurring organic compounds which contain bromine.

The hydroxyflavone glycosides contain flavonol, or hydroxyflavone, or some derivative of this, as the non-sugar radical



Flavonol

All the flavonol derivatives are yellow dyes. In many of these glycosides the carbohydrate is rhamnose. Quercitrin occurs in oak bark. On hydrolysis it yields quercetin (1.3.3′4′-tetrahydroxyflavonol) and rhamnose. Such glycosides are usually the raw materials for the preparation of rhamnose.[8] Quercetin is widespread as a plant pigment, occurring in red onion skins, cotton flower, etc.

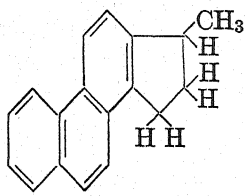
SAPONINS.—The saponins comprise a large group of compounds widely distributed in the higher plants. They are mostly amorphous substances, soluble in water to colloidal solutions which foam readily and which stabilize emulsions of fats and oils, so that such emulsions are very difficult to "break." The name signifies that they are "soap-like." They greatly reduce surface tension. The "soap weeds," "soap bushes," etc., all owe their detergent effect to saponins. In certain instances they have been used to furnish foam on manufactured beverages. This is, however, now generally prohibited by law because of the undesirable physiological action of the saponins.

On hydrolysis with mineral acids they yield sugars, such as glucose, galactose, arabinose, and rhamnose, together with "sapogenins,"

[7] Friedländer, P., Über schwefelhaltige Analoga der Indigogruppe, *Ber.*, 39: 1060–1066 (1906); Über den Farbstoff des antiken Purpurs aus Murex brandaris, *Ber.*, 42: 765–770 (1909).

[8] Walton, C. F., Jr., The Preparation of Rhamnose, *J. Am. Chem. Soc.*, 43: 127–131 (1921).

or closely related compounds which are derivatives of the reduced cyclopentenophenanthrene nucleus:

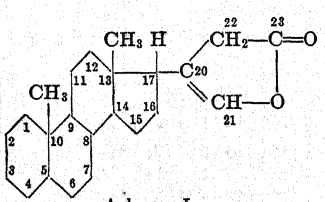


γ-Methyl-cyclopentenophenanthrene
(Diels hydrocarbon)

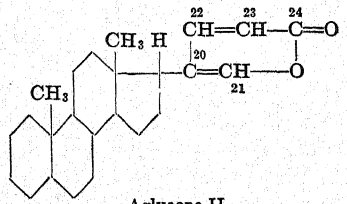
This hydrocarbon, which is known as Diels hydrocarbon, is obtained by the dehydrogenation of cholesterol, ergosterol, the sex hormones, the toad poisons (bufotoxin, bufotalin, and cinobufagin), and the non-sugar constituent of the cardiac glycosides and of the sapogenins. Likewise the non-sugar residue of the poisonous glycosides present in squills contains this nucleus, and it is also the characteristic nucleus of the carcinogenetic compounds. Therefore this nucleus has not only a wide distribution in nature but is the basis of a great many physiologically active compounds.

Phenanthrene chemistry, so far as it relates to the structure of these important physiological compounds, dates almost exclusively since 1920, and most of the structural problems have been elucidated since 1930. The recent American Chemical Society Monograph of Fieser [9] sums up the literature. The first edition was published in 1936 and summed up the literature to the end of 1935. A second edition was issued a year later and necessitated an expansion of 91 pages to cover the literature of 1936, in which year more than 300 papers in the field of phenanthrene chemistry appeared.

While the cyclopentenophenanthrene nucleus is the characteristic nucleus of the physiologically active compounds noted, the actual compounds contain the *hydrogenated* nucleus and are derivatives of the ring structures designated as aglycone I, aglycone II, or the sapogenin nucleus.

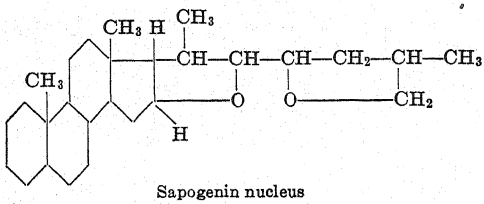


Aglycone I



Aglycone II
(Scillaridin-A nucleus)

[9] Fieser, L. F., The Chemistry of Natural Products Related to Phenanthrene, Second Edition, A. C. S. Monograph 70, 456 pages, Reinhold Publishing Corporation, New York (1937).



Sapogenin nucleus

From the medical standpoint the most important group of glycosides which are usually classed with the saponins are those which occur in the foxglove, *Digitalis purpurea*. It is generally stated that this plant contains at least five glycosides. It has been shown, however, that certain of these are decomposition products and that it contains three distinct glycosides designated as digilanide-A, digilanide-B, and digilanide-C, in which the non-sugar residue is, respectively, digitoxigenin, gitoxigenin, and digoxigenin. In addition digitalis extract contains the three saponins, digitonin, gitonin, and tigonin, which break down respectively to yield the sapogenins, digitogenin, gitogenin, and tigogenin. The relationship of these compounds to the structures of aglycone I, aglycone II, and the sapogenin nucleus is shown in Table LXXV.

TABLE LXXV

STRUCTURE OF THE NON-SUGAR RESIDUES IN CERTAIN CARDIAC GLYCOSIDES AND SAPONINS

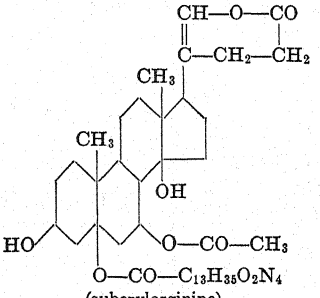
Substance	Empirical Formula	Structure
Digitoxigenin	$C_{23}H_{34}O_4$	3-14-dihydroxyaglycone I
Gitoxigenin	$C_{23}H_{34}O_5$	3-14-16-trihydroxyaglycone I
Digoxigenin	$C_{23}H_{34}O_5$	3-11-14-trihydroxyaglycone I
Digitogenin	$C_{27}H_{44}O_5$	2-3-6-trihydroxysapogenin
Gitogenin	$C_{27}H_{44}O_4$	2-3-dihydroxysapogenin
Tigogenin	$C_{27}H_{44}O_3$	3-hydroxysapogenin
Sarsasapogenin	$C_{27}H_{44}O_3$	11-hydroxysapogenin
Scillaridin A	$C_{24}H_{30}O_3$	14-hydroxyaglycone II

Digitalis extract is a unique drug in respect to cardiac action. It decreases the frequency and strengthens the intensity of the heart beat. No natural or synthetic drugs have been found to replace digitalis in medicine.

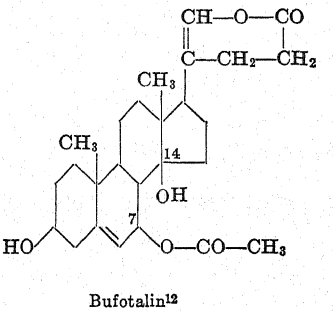
The saponin digitonin is the major component of the glycosides of digitalis. It possesses the valuable property of forming an insoluble compound with cholesterol and other sterols and is used for the quan-

titative estimation of these compounds.[10] The digitonin may be later recovered [11] from the sterol-digitonin precipitate.

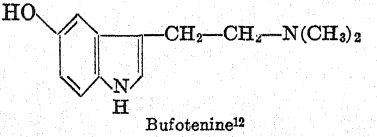
The fish poisons of the aborigines, particularly of Africa and South America, are saponins containing the phenanthrene nucleus. Plants containing these compounds are macerated and thrown into the stream, and the fish which are poisoned rise to the surface. The saponins show a hemolytic action on erythrocytes, possibly owing to the withdrawal of cholesterol from the corpuscle wall. The toad poisons likewise are derivatives of the phenanthrene nucleus. In *Bufo vulgaris* the poisonous glands are situated back of the eyes and the secretion is a somewhat milky fluid containing bufotoxin, bufotalin, bufotenine, and epinephrine. Bufotoxin is a cardiac poison more or less similar in its action to digitalis extract but lacking the persistency of action which characterizes the digitalis glycosides. Bufotenine is unusual in that it is a derivative of 5-hydroxyindole. It is a cardiac stimulant and shows pressor action. Bufotoxin shows the unusual grouping of an esterification with suberylarginine on the hydroxyl group situated on carbon-5 of the phenanthrene nucleus.



(suberylarginine)
Bufotoxin[12]



Bufotalin[12]



Bufotenine[12]

[10] Windaus, A., Über die quantitative Bestimmung des Cholesterins und der Cholesterinester in einigen normalen und pathologischen Nieren, *Z. physiol. Chem.*, 65: 110–117 (1910); Windaus, A., and Shah, S. V., Über einige neue Abbauprodukte des Digitogenins, *Z. physiol. Chem.*, 151: 86–97 (1926); *cf.* also Palmer, L. S., and Eckles, C. H., Carotin—The Principal Natural Yellow Pigment of Milk Fat—Pt. II, *Research Bull.* 10, Missouri Agr. Exp. Sta. (1914).

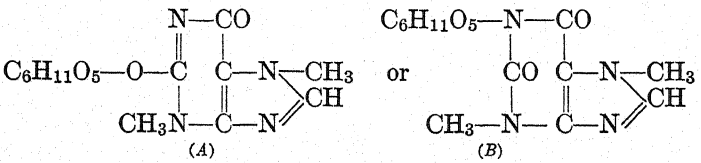
[11] Panzer, T., Notizen über die chemische Zusammensetzung der Tuberkelbazillen, *Z. physiol. Chem.*, 78: 414–419 (1912).

[12] Formulas from Wieland and Hesse, *Ann.* 517: 22 (1935).

SYNTHESIS OF GLUCOSIDES.—If we add the sodium or potassium salt of a phenol to acetobromglucose or acetochlorglucose (from a pentaacetyl sugar treated with anhydrous HCl or HBr,[13] the Cl or Br replaces the —OH group on carbon-1), we obtain the corresponding phenol derivative. The acetyl groups are then removed by hydrolyzing with dilute alkali, resulting in the formation of the glucoside. A number of the simpler natural glucosides have been synthesized by the above or similar methods.

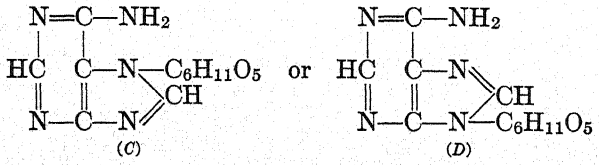
Fischer synthesized certain of the α-series and found that none of these were hydrolyzed by emulsin. He[14] also prepared glucosides, galactosides, and rhamnosides of the purines, *e.g.*, adenine, guanine, xanthine, hypoxanthine, and theobromine. In this instance the compounds which are formed may be somewhat different in structure from the naturally occurring glycosides, which have already been considered, in that the linkage from the sugar to the non-sugar may not be through an oxygen but rather may be a direct bond from carbon to nitrogen.

Thus, for theobromine we have the possibilities of either



In (*A*) we have a true glycosidal linkage through oxygen, whereas in (*B*) the union is from carbon to nitrogen. Fischer was unable to decide which of these formulas represented the compound which was isolated.

In compounds where the purine or pyrimidine does not contain oxygen, *e.g.*, adenine, there is no possibility of the true glycosidal linkage. In this instance, Fischer presents the alternative formulas,

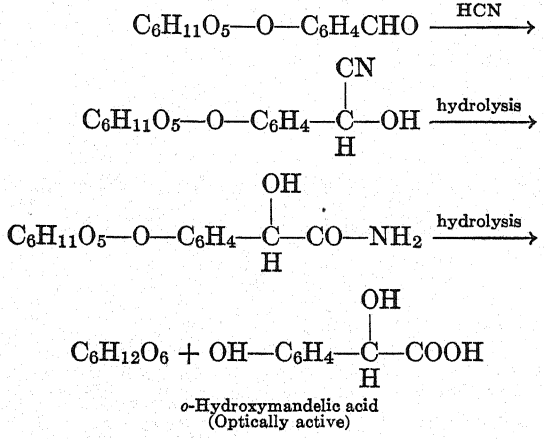


[13] Fischer, E., and Armstrong, E. F., Über die isomeren Acetohalogen-Derivate des Traubenzuckers und die Synthese der Glucoside, I–III, *Ber.*, 34: 2885–2900 (1901); 35: 833–843 (1902); 35: 3153–3155 (1902).

[14] Fischer, E., and Helferich, B., Synthetische Glucoside der Purine, *Ber.*, 47: 210–235 (1914); Fischer, E., and Fodor, K. v., Notiz über Theophyllin-rhamnosid, *Ber.*, 47: 1058–1061 (1914); *cf.* also Helferich, B., and Kühlewein, M. v., Synthese einiger Purin-glucoside, *Ber.*, 53: 17–22 (1920).

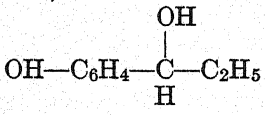
and notes that it was impossible to distinguish with certainty between these formulas, although he was inclined to regard formula (*C*) as the more probable. Such compounds are probably very closely related to the nucleosides derived from nucleic acid, except that *d*-ribose is the sugar present in the nucleosides. As a matter of fact, Fischer's [15] phosphoric acid esters of the purine glycosides may be regarded as synthetic nucleotides.

ASYMMETRIC SYNTHESES.—Fischer suggests that the optical activity of the sugar molecule may be responsible for the formation in nature of various optically active components on the hypothesis that the optically active compounds are perhaps originally associated with a sugar molecule and later are hydrolyzed off. Fischer and Slimmer [16] have presented certain proof for this hypothesis. Thus, using the glycoside, *helicin*, in which the non-sugar radical is salicylic aldehyde, they found that it was possible to prepare the cyanhydrin by the addition of HCN which could be later hydrolyzed to the corresponding acid amide. On further hydrolysis the sugar molecule was split off, yielding an optically active orthohydroxymandelic acid,



o-Hydroxymandelic acid
(Optically active)

In a somewhat analogous manner, the same authors prepared orthohydroxyphenylethylcarbinol,



which again showed a relatively high specific optical rotation, −9.83°.

[15] Fischer, E., Über Phosphorsäureester des Methyl-glucosids und Theophyllin-glucosids, *Ber.*, 47: 3193–3205 (1914).

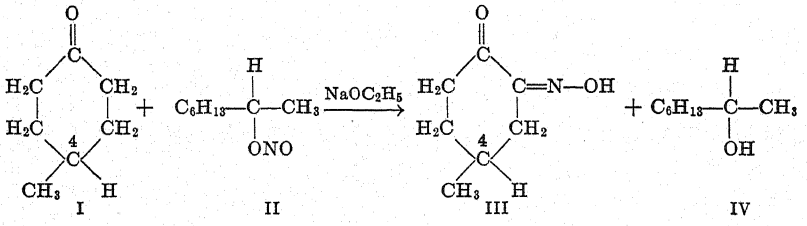
[16] Fischer, E., and Slimmer, M., Versuche über asymmetrische Synthese, *Ber.*, 36: 2575–2587 (1903).

Fischer notes that he could obtain no evidence for the formation of more than one of the optically active isomers, and in these instances at least there is no possibility that α- and β-forms were originally present to account for the asymmetric synthesis, inasmuch as the glucose radical was originally present entirely in the β-form and in itself did not enter into the synthetic reactions, although it must have influenced them.

Most asymmetric syntheses which have been reported involve the creation of an asymmetric carbon atom during the synthetic reactions. Pezold and Shriner [17] report an asymmetric synthesis where the asymmetric carbon atom was already present in the molecule prior to the series of synthetic reactions. The reactions studied involved the formation of an α-oximinoketone by the action of a nitrite on a ketone.

$$RCOCH_2R + R'ONO \rightarrow \underset{\underset{N=O}{|}}{RCOCHR} \rightleftarrows \underset{\underset{N-OH}{|}}{RCOCR} + R'OH$$

The specific reaction studied was the treatment of 4-methylcyclohexanone with optically active *d*- and *l*-2-octylnitrite in an alcohol-ether solution yielding an oxime having the formula III, and converting the octylnitrite into a secondary alcohol.



Carbon-4 of the original ketone was not an asymmetric carbon, but when the ketone is transformed into the oxime, carbon-4 becomes asymmetric. When *d*-2-octylnitrite was used in this reaction, the oxime (III) which was formed was optically active and *levorotatory*, the octanol-2 (IV) was dextrorotatory. When *l*-2-octylnitrite was used, the oxime was *dextrorotatory*, but the secondary octyl alcohol was levorotatory. When *d-l*-2-octylnitrite was used, the synthesized oxime and alcohol showed no optical activity. Whether or not this reaction has any analogies in the reactions which occur in nature is unknown, but it does demonstrate the influence of one optically active compound on the configuration of the asymmetric carbon atom in another compound.

[17] Pezold, Margaret, and Shriner, R. L., Asymmetric Syntheses. I. The Action of Optically Active Nitrites on Cyclic Ketones, *J. Am. Chem. Soc.*, 54: 4707–4711 (1932).

It seems to be generally established that organic syntheses are likely to result in an asymmetric synthesis if the chemical reactions take place in the presence of other optically active organic compounds. From a philosophical standpoint at least the question now arises as to how an asymmetric synthesis originated in nature. Oparin[18] discusses this subject in his fascinating consideration of "The Origin of Life." He points out that sky light is partially plane polarized and that when such light is reflected from the surface of water, it is transformed into elliptically polarized light. He accordingly reaches the conclusion that an excess of one or the other optical antimeres was produced by a photochemical reaction under the influence of elliptically polarized light. That this assumption has an experimental basis is indicated by the observations of Kuhn[19] and Mitchell.[20]. Kuhn observed that *d-l*-ethyl-α-brompropionate had a strong absorption band in the ultraviolet at 2800 Å. He rayed this compound dissolved in alcohol with *dextro* circularly polarized light and found that the *l*-form was more rapidly destroyed than was the *d*-form, so that an excess of the *d*-ethyl-α-brompropionate remained in solution. When *levo* circularly polarized light was used, the residual solution showed an excess of the *levo* compound in solution.

Later Kuhn used *d-l*-α-azidopropiondimethylamide which has its absorption band at 2900 Å. Again, this compound was found to decompose in hexane solution by light of 2900 Å and to liberate N_2 with a quantum efficiency of unity. Again, *dextro* circularly polarized light decomposed the *l*-form more rapidly than the *d*-form and *vice versa*, so that optically active residues resulted. Mitchell used *d-l*-humulene nitrosite which contains the grouping:

$$(CH_3)_2\text{—}C(ONO)\text{—}\underset{*}{C}(NO)\begin{matrix}R\\ \diagdown\\ \diagup\\ R'\end{matrix}$$

where the carbon which is starred is asymmetric. The *d-l*-compound was dissolved in ethyl butyrate and rayed with *dextro* and *levo* circularly polarized light of wave length of about 7000 Å where this compound had a pronounced absorption band. Again, the *dextro* circularly polarized light decomposed the *l*-form more rapidly than the *d*-form, resulting in a readily detectable residual *d*-rotation, and the *levo* circularly polarized light had the opposite effect and produced an excess of the *l*-form.

[18] Oparin, A. I., The Origin of Life, Translated by S. Morgulis, The Macmillan Company, 1938. (Every student interested in biological phenomena should read this volume.)

[19] Kuhn, W., and Knopf, E., Darstellung optisch activer Stoffe mit Hilfe von Licht, *Z. physik. Chem.*, B7: 229–310 (1930); *cf.* also *Naturwissenschaften*, 17: 227–228 (1929); 18: 183 (1930).

[20] Mitchell, S., The Asymmetric Photochemical Decomposition of Humulene Nitrosite by Circularly Polarised Light, *J. Chem. Soc.*, 1829–1834 (1930).

CHAPTER XXVIII

THE PECTIC SUBSTANCES*

The term "pectin" is used to denote the substance or substances which, in the presence of the proper concentrations of acid and a sugar, will form the familiar fruit jellies and jams. The pectin producing these jellies is the only water-soluble member of a group of related compounds known as the "pectic substances," or sometimes called the "pectins." At the present time three of these substances are generally recognized: *protopectin, pectin,* and *pectic acid.*

The pectic substances are colloidal carbohydrates of high molecular weight and rather complex composition. Galacturonic acid, galactose, arabinose, xylose, methanol, and acetic acid have been identified as hydrolytic products. However, as the presence of both galactose and arabinose has been questioned, a brief summary of the evidence for each substance may be desirable.

Galacturonic acid was isolated by Ehrlich [1] from beet pectin after hydrolysis of the latter with oxalic acid. As already indicated in the discussion of the uronic acids, it is probably formed in the plant by oxidation of the primary alcohol group of galactose. On treatment with nitric acid, the galacturonic acid is oxidized to mucic acid. It has long been known that mucic acid is one of the products which can be obtained from pectin treated with nitric acid. Arabinose is formed by decarboxylation of the galacturonic acid. Decarboxylation may be accomplished by boiling with 12 per cent hydrochloric acid,[2,3] or less readily by heating with alkali.[4]

The formation of carbon dioxide when pectins were heated with acid indicated the presence of uronic acids long before Ehrlich iso-

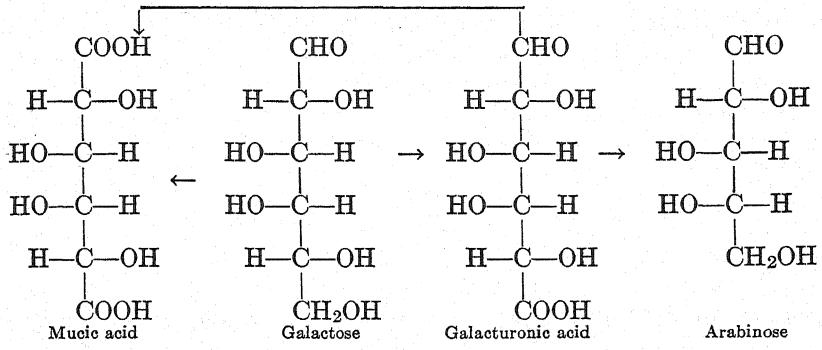
*This chapter is contributed by Dr. J. J. Willaman, Biochemist, Rohm and Haas Co., Inc., Philadelphia, formerly Professor of Agricultural Biochemistry in the University of Minnesota.

[1] Ehrlich, F., Die Pektinstoffe, Ihre Konstitution und Bedeutung, *Chem. Ztg.*, 41: 197–200 (1917).

[2] Lefèvre, K. U., and Tollens, B., Untersuchungen über die Glucuronsäure, ihre quantitative Bestimmung und ihre Farbenreaktionen, *Ber.*, 40: 4513–4523 (1913).

[3] Nanji, D. R., Paton, F. J., and Ling, A. R., Decarboxylation of Polysaccharide Acids; Its Application to the Establishment of the Constitution of Pectins and to Their Determination, *J. Soc. Chem. Ind.*, 44: 253–258T (1925).

[4] Candlin, E. J., and Schryver, S. B., Investigations of the Cell-Wall Substances of Plants, with Special Reference to the Chemical Changes Taking Place During Lignification, *Proc. Roy. Soc.* (*London*), 103B: 365–376 (1928).



lated galacturonic acid. We now know that the latter occurs to the extent of 70 to 80 per cent in the various pectic substances.

Galactose has been recognized for a long time as a constituent of the pectins. Its presence was at first merely surmised from the fact that mucic acid is formed on treatment of pectins with nitric acid. Since, however, galacturonic acid also yields mucic acid, the isolation of the sugar from the hydrolytic products of the pectin furnished the necessary proof. It is unlikely that it is produced from any other substance during hydrolysis by weak acids.

On the other hand, there has been real reason to question the presence of arabinose. It was at first surmised from the fact that the pectic substances give very strong reactions for pentoses; in fact, by the phloroglucide method of pentose estimation, a very high percentage of pentose in the pectin can be demonstrated. However, in this method furfural is formed by boiling the material with 12 per cent hydrochloric acid. This treatment converts the galacturonic acid to arabinose, and then the latter to furfural. Hence, this phloroglucide method is not necessarily either a qualitative or a quantitative indication of pentose. Furthermore, although arabinose has been identified as such in the acid hydrolysate of pectins, it could easily be an artifact, due to decarboxylation of some of the galacturonic acid. Recently, Myers and Baker [5] have apparently succeeded in avoiding these difficulties and have established the presence of arabinose in citrus pectin.

Methanol is a characteristic constituent of both pectin and protopectin. Fellenberg [6] showed that it is combined with pectin in ester linkages, and can be easily removed by saponification. Later work makes it seem probable that it is esterified with the carboxyl groups of the galacturonic acids.

Acetic acid has been definitely identified in the pectin of flax and

[5] Myers, P. B., and Baker, G. L., Fruit Jellies. VIII. The Rôle of Pectin. 4. The Physico-chemical Properties of Pectin, Bul. 187, Delaware Agr. Exp. Sta. (1934).

[6] Fellenberg, Th. von, Über die Konstitution der Pektinkörper, *Biochem. Z.*, 85: 118–161 (1918).

of sugar beet by Ehrlich,[7,8] of sugar beet by Nelson,[9] and of lemons by Myers and Baker.[5] Nelson could not find it in the pectin from the fruit of apple, tomato, or lemon.

CONSTITUTION OF THE PECTIC SUBSTANCES.—With the above brief enumeration of the constituent groups found in the pectins, we are in a position to discuss how these groups are combined in the various individual pectic substances. Protopectin, as its name implies, is the mother substance of this group. It occurs in the cell walls of most plant tissues. The older name for this substance was pectose.

There are two places in plant cell walls where pectic compounds exist. Pectin compounds form the middle lamella, where they act as a cementing material between cells, and they likewise occur as incrustations or thickenings on the cell wall. There has been considerable shifting of opinion concerning the nature of the pectin compounds in these two regions. In the early history of pectin chemistry their identity was kept separate, and the existence of still other pectic compounds was hypothecated. Later it was thought simpler to consider all insoluble cell-wall pectin as pectose. Still later this name was changed to protopectin. And at the present time there is very good evidence adduced to show that the cell-wall thickenings and the middle lamella are quite different in nature.[10, 11] Carré and Haynes call the material forming the thickenings "pectose," and the other "middle-lamella pectin." Which of these should be called "protopectin," as the precursor of pectin, cannot be decided with our present knowledge. There is much evidence that both these substances are combined with cellulose; that cellulose is actually esterified with some of the galacturonic acid groups; that in the middle-lamella pectin there is more cellulose and less methoxy groups, and in the cell-wall thickenings there is less cellulose and more methoxy groups in combination with the pectin radical. Thus, Schweitzer's reagent will completely dissolve away all cellulose substances and leave the middle lamella as a fine network. This network then readily dissolves in weak acid followed by weak alkali. Sucharipa [12] has prepared a material by Schweitzer's reagent which he believes to be protopectin.

[7] Ehrlich, F., and Sommerfeld, R. von, Die Zusammensetzung der Pektinstoffe der Zuckerrübe, *Biochem. Z.*, 168: 263–323 (1926).

[8] Ehrlich, F., and Schubert, F., Über die Chemie der Inkrusten des Flachses, *Biochem. Z.*, 169: 13–66 (1926).

[9] Nelson, E. K., Acetyl Groups in Pectin, *J. Am. Chem. Soc.*, 48: 2945–2946 (1926).

[10] Carré, M. H., and Haynes, D., The Estimation of Pectin as Calcium Pectate and the Application of This Method to the Determination of the Soluble Pectin in Apples, *Biochem. J.*, 16: 60–69 (1922).

[11] Carré, M. H., Chemical Studies in the Physiology of Apples. IV. Investigations on the Pectic Constituents of Apples, *Ann. Botany*, 39: 811–839 (1925).

[12] Sucharipa, R., Protopectin and Some Other Constituents of Lemon Peel, *J. Am. Chem. Soc.*, 46: 145–156 (1924).

In beet root, at least,[7] protopectin consists of an araban plus the Ca-Mg salt of pectin, combined with the cell wall. On hydrolysis the two constituents are rendered soluble, and then the araban can be separated from the Ca-Mg pectin by 70 per cent alcohol.

Protopectin can be hydrolyzed free from the cellulose of the wall and converted into soluble "pectin" by several means: (1) by long-continued boiling with water, especially under pressure; (2) by treatment with 0.5 per cent ammonium oxalate at 70–90°; (3) by action of the enzyme protopectinase; (4) by heating with dilute acids; and (5) by electrodialysis.[13] The second method has been the one most frequently employed in the laboratory, although the fourth method is the commercial one. The transformation also occurs during the ripening of fruits. However it is brought about, it results in the separation of the cells from one another and is usually spoken of as "maceration."

The resultant pectin is the best known of all the pectic substances, and is the pectin of commerce. It is a material of high molecular weight; it disperses in water to a viscous colloidal sol; it is readily precipitated from this sol by alcohol, which acts as a dehydrating agent, by lead, iron, and other heavy metal salts, but not by salts of calcium. Its most outstanding property is its ability to form sugar-acid-pectin gels, and such fruit jellies have been long known.

The purest preparations of pectin contain from 10 to 12 per cent of methyl alcohol combined as methoxy groups or as methyl ester groups. Demethoxylation occurs slowly when the pectin is boiled with water or dilute acid; but it occurs within a few minutes at room temperatures when pectin is treated with dilute alkali. In fact, this saponification constitutes a simple method for determining the methoxy content.

This demethoxylated pectin is pectic acid.[14] Probably the purest preparations of pectic substances so far described have been of this pectic acid, because of the facts that it is a simpler substance and that its solubilities enable it to be purified more readily. The usual procedure is to prepare pectin by repeated precipitations with alcohol; saponify the pectin with dilute sodium hydroxide, producing soluble sodium pectate; acidify with hydrochloric acid, producing the gelatinous, insoluble pectic acid; and dry this with alcohol and ether. Or the saponification may be brought about by limewater, producing the gelatinous, insoluble calcium pectate. This gel may be freed from calcium by treating with ammonium oxalate, inasmuch as the ammonium pectate is soluble.

Views on the structure of pectic acid have undergone a series of

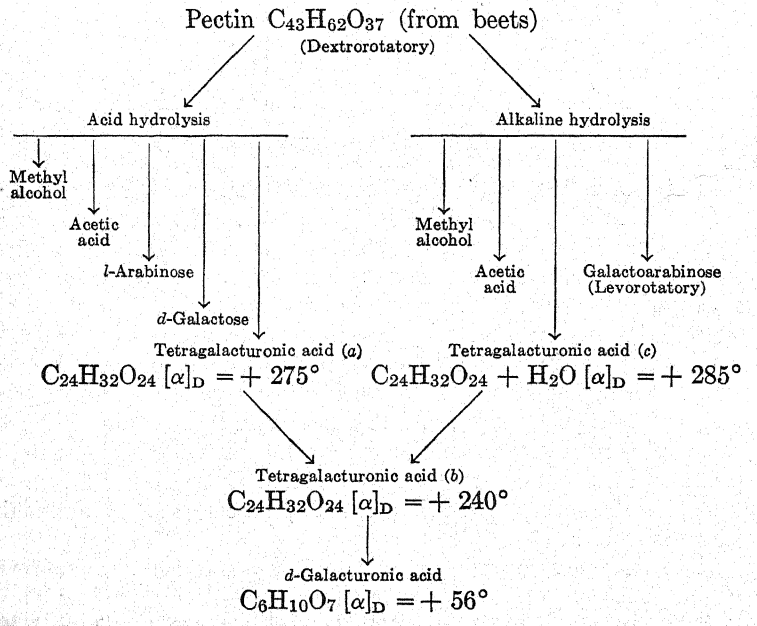
[13] Gortner, R. A., and Hoffman, W. F., Extracting Pectin-like Substances from Materials such as Fruits and Vegetables by Electrodialysis, U. S. Patent 1,915,568, June 27, 1933.

[14] The term, "pectinic acids," has been revived by Carré to apply to compounds of intermediate methoxy content, *i.e.*, between pectin and pectic acid.

changes. In 1916, Schryver and Haynes[15] prepared pectic acid from four different sources, and found that all four agreed with the empirical formula $(C_{17}H_{24}^{*}O_{16})_x$. Later Carré and Haynes prepared a calcium pectate that agreed well with this formula. Then, Nanji, Paton, and Ling isolated what they consider to be the basic unit of the pectins, a substance with the empirical formula $(C_{35}H_{50}O_{33})_x$. This again agrees well with Schryver and Haynes' formula. Furthermore, from analysis of the constituent sugars and galacturonic acid they proposed the following structure for pectic acid: four molecules of galacturonic acid, one of galactose, and one of arabinose, arranged in a ring, with the carboxyls of the acid groups free and available for methyl ester formation. The specific order of the units in the ring was not suggested.

Ehrlich,[16] after careful work on beet and flax pectin covering several years, found the same constituents, and in the same proportions. However, he isolated several tetragalacturonic acids of closed-ring structure, which led him to the conclusion that the sugar acids and sugars are not in the same ring.

Ehrlich diagrams the decomposition products of beet pectin as follows:



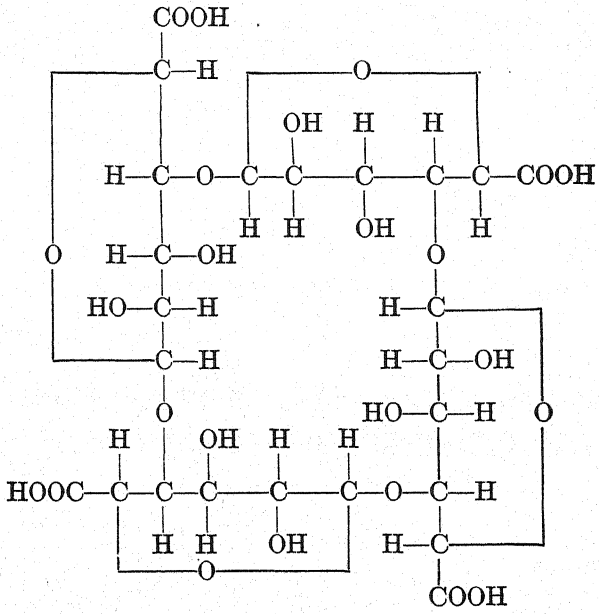
[15] Schryver, S. B., and Haynes, D., The Pectic Substances of Plants, *Biochem. J.*, 10: 539–547 (1916).

[16] Ehrlich, F., Neue Untersuchungen über Pektinstoffe, *Z. angew. Chem.*, 40: 1305–1313 (1927).

The galactose-arabinose disaccharide was isolated, indicating that both sugars are not connected directly to the ring.

On this basis the nucleus of pectic acid appears to be a new type of carbohydrate derivative, *i.e.*, a tetragalacturonic acid in which four molecules of galacturonic acid are condensed with the elimination of four molecules of water. Each of the four aldehyde groups is linked to a hydroxyl group in another molecule, and all four of the carboxyl groups remain free to be esterified with methyl alcohol or to form metallic salts. The galactoarabinose disaccharide appears to be linked to the tetragalacturonic acid through one of the hydroxyls of the latter.

Such a nucleus would have approximately the following formula:



Ehrlich's tetragalacturonic acid, $C_{24}H_{32}O_{24}$
(position of oxygen linkages not certain)

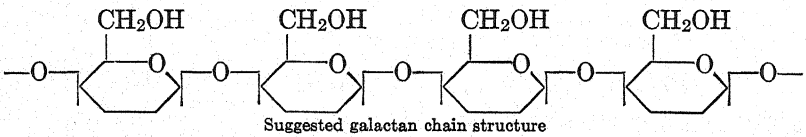
More recently Myers and Baker [5] have deduced evidence that pectic acid consists of two closed rings of tetragalacturonic acid joined together: or a total of eight acid groups minus nine molecules of water, with all carboxyl groups exposed.

It should be stressed here that pectic acid is the nucleus of all the pectic substances: that pectin and protopectin consist of this nucleus combined with the various other materials which have been found—methanol, acetic acid, galactose, arabinose, xylose, araban, cellulose, possibly lignin. With this in mind, we are now in a position to consider the most likely formulas for pectin that have been proposed.

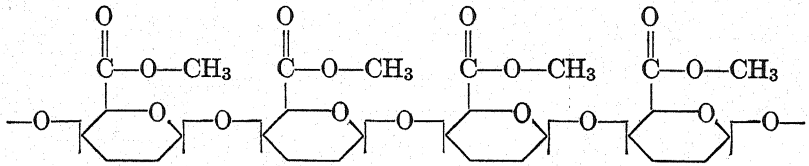
Ehrlich believes that the pectin from sugar beets is a triacetylarabinogalactodimethoxytetragalacturonic acid ($C_{43}H_{62}O_{37}$). The pectin from flax straw differs from this in having but two molecules of acetic acid, and in having in addition one molecule of xylose. This pectin he designates as diacetylarabinoxylogalactodimethoxytetragalacturonic acid ($C_{46}H_{68}O_{40}$). The positions of attachment of these various molecules have not been determined.

Myers and Baker [5] submit that lemon pectin is monoarabinomonogalactodiacetylheptamethoxyloctagalacturonic acid. Seven of the eight carboxyls of the octa acid are methoxylated, the eighth one is free. Assuming the elimination of 20 molecules of water in the above complex, the formula for pectin would be $C_{70}H_{98}O_{58}$, with a molecular weight of 1,866. Confirmatory evidence that the polygalacturonide nucleus of pectin contains from 8 to 12 galacturonic acids was obtained by Baur and Link [17] by an entirely different procedure.

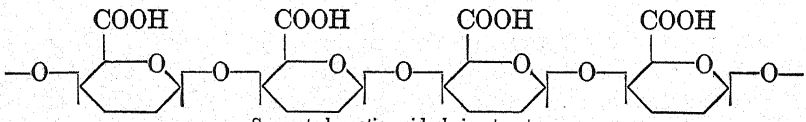
Bonner [18] argues for a long cellulose-like chain of galacturonic acid units arranged more or less as the galactose units are arranged in galactan except that carbon-6 is in a carboxyl group in the pectins instead of carrying the normal primary alcohol group characteristic of the sugars. Occasionally a galactose residue or an arabinose residue may replace a galacturonic acid residue in the chain, but such replacement would not disturb the normal linkage relationships.



Suggested galactan chain structure



Suggested pectin chain structure



Suggested pectic acid chain structure

Bonner bases his argument for the "cellulose" or "galactan" chain type of structure for the pectins on (*a*) the X-ray diffraction studies

[17] Baur, L., and Link, K. P., The Methylglycosides of the Naturally Occurring Hexuronic Acids, IV, *J. Biol. Chem.*, 109: 293–299 (1935).

[18] Bonner, J., The Chemistry and Physiology of the Pectins, *Botan. Rev.*, 10: 475–497 (1936).

of Meyer and Mark [19] and of Van Iterson [20] who have shown that the galacturonic residues are arranged in chains and (*b*) the viscosity studies of Henglein and Schneider [21] which indicate that at least two hundred galacturonic residues are present in the average chain. These last workers nitrated pectin and pectic acid and found that the nitrated products had physical properties closely analogous to those of nitrocellulose and that the nitrated pectic acid had a particle weight of the order of 20,000. It contained two nitro groups instead of the three which characterize cellulose. Its chief contrast in behavior to nitrocellulose was its solubility in strong alkalies—by virtue of the free carboxyl groups in the molecule.

Bonner [22] further points out that pectic acid possesses a very high charge density and that its negative charge is due almost entirely to the free carboxyl groups. In a "calcium pectate" one calcium ion is shared between two carboxyl groups, the calcium ion thus serving, through electrostatic attraction, to hold two chains of the pectic acid more or less parallel to each other, so that dried calcium pectate shows orientation and in many instances is doubly refracting. The "equivalent weight" per equivalent (−) charge lies between 203 and 229.

Bonner points out that pectin sols differ from most hydrophilic sols by the fact that the micelles are larger, in reality gel fragments, and that this is the reason that pectin is so readily precipitated from the sol by dehydrating agents.

Apparently Norman [23] agrees with Bonner that the tetragalacturonic acid theory of Ehrlich or the hexa ring of Nanji, Paton, and Ling are incompatible with our present knowledge, and he appears to favor a longer polygalacturonide chain structure, although he notes that there is one unique property of pectin in which it differs markedly from other polysaccharides, such as starch, cellulose, or the hemicelluloses, *i.e.*, that pectin is extremely sensitive toward hot alkali and that even such dilute solutions as 0.2 per cent sodium hydroxide will degrade pectin in 10 minutes so that more than 70 per cent of the pectin is hydrolyzed beyond the calcium pectate stage. Norman points out that no evidence is available in any postulated chemical structure which will account for its sensitivity toward dilute alkali.

[19] Meyer, K. H., and Mark, H., Der Aufbau der hochpolymeren organischen Naturstoffe, Akademische Verlagsgesellschaft, Leipzig (1930).

[20] Van Iterson, G., Jr., Biologische inleidung tot het cellulose-symposium, *Chem. Weekblad*, 30: 2–19 (1933).

[21] Henglein, F., and Schneider, G., Über die Veresterung von Pektinstoffe, *Ber.*, 69B: 309–324 (1936).

[22] Bonner, J., Some Colloidal Properties of the Pectins, *Proc. Acad. Sci. Amsterdam*, 38: 346–354 (1935).

[23] Norman, A. G., The Biochemistry of Cellulose, the Polyuronides, Lignin, etc., Oxford University Press, England (1937).

The important point of comparison in the studies on pectin made by the various workers is not in the disagreement with regard to structure, but in the really close agreement in the kind and quantity of constituent groups. This augurs well for the future elucidation of the structure of the pectic substances.

COMMERCIAL PECTIN.—The introduction of commercial pectin has made possible the production, either in the home or in the factory, of jellies from fruits which contain too little pectin of their own; it has made possible the standardization of jelly making; and it eliminates the long cooking of delicately flavored fruits.

Most commercial pectin is prepared either from apple pomace or cull lemons. Table LXXVI shows the quantities of pectin in these

TABLE LXXVI

PERCENTAGE OF PECTIN IN RAW MATERIALS

	Fresh Material	Dry Matter Basis
Apple pomace	1.5–2.5	15.0–18.0
Lemon pulp	2.5–4.0	30.0–35.0
Orange pulp	3.5–5.5	30.0–40.0
Beet pulp	1.0	25.0–30.0
Carrots	0.62	7.14

and in other materials suggested as sources of pectin.[24] The pulp, either apple or lemon, is given a preliminary washing, and is then cooked with dilute acid, either mineral or organic, usually together with sulfurous acid, to bring the pectin into solution. The filtered extract may be evaporated to a syrup and sold as liquid pectin, or the pectin may be precipitated by various means. In the case of lemon albedo extracts the pectin is precipitated with aluminum sulfate and ammonia.[24] This precipitate is dried and the aluminum removed by washing with acidified alcohol. The final product is sold as powdered pectin. Another method is the precipitation of the pectin by alcohol. Still another is the drying of the pectin sol in a thin film on a drum.[25]

Pectin is usually sold according to its "jelly grade," which is the number of pounds of sucrose that 1 pound of pectin can "carry" in a jelly of standard acidity and water content. This jelly grade is usually not above 150 in commercial pectin, but 514 has been attained in the

[24] Wilson, C. P., The Manufacture of Pectin, *Ind. Eng. Chem.*, 17: 1065–1067 (1925).

[25] Cowgill, W. W., Pectin, U. S. Patent 1,973,614.

laboratory, with indications of 520 as a theoretical limit for lemon pectin.[26]

The quantitative extraction of pectin from lemon albedo (white portion of rind), and the factors affecting the jelly grade of pectin, have been exhaustively investigated by Myers and Baker.[26] The *p*H, temperature, and time of extraction are closely interdependent in controlling the yield of pectin and the jelly grade. Thus, with 30 minutes of heating, 80° C. and *p*H 1.5 are optimal. With increasing time of heating the yield of pectin increases, but the jelly grade decreases, and so on through other combinations. The manufacturer thus has a choice of factors with which to work out the most economical conditions for production. Myers and Baker have further found that the relative viscosity of pectin solutions is a measure of their grade up to a value of 350, and that a simple viscosity measurement with a pipet will tell the sugar-holding capacity of a fruit juice.[27] The viscosity, and hence the jelly grade, is conditioned by the degree of polymerization of the polygalacturonic acid residues. The methoxy content, and that of some of the other constituents, may be considerably reduced without impairing the viscosity, provided depolymerization does not occur.

In recent years the manufacture of pectin has steadily increased, and new uses are continually sought for it. Some proposed uses are: in case hardening of metals, as non-hygroscopic agent in dried fruit juices, in sizing of textiles, as emulsifying agent, in bakery products to delay staling, in pharmaceutical salves.

A gel made with pectin requires three ingredients in proper concentration—pectin, sugar (usually sucrose), and acid. The sugar must be present to the extent of 65 to 70 per cent, and the acidity must be equivalent to a *p*H of 3.2 to 3.5.[28-32] This acidity is attained by the

[26] Myers, P. B., and Baker, G. L., Fruit Jellies. VII. Role of Pectin. 3. Effect of Temperature upon the Extraction of Pectin. Bull. 168, Delaware Agr. Exp. Sta. (1931).

[27] Myers, P. B., and Baker, G. L., Fruit Jellies. V. The Role of Pectin. I. The Viscosity and Jellying Properties of Pectin Solutions, Bull. 149, Delaware Agr. Exp. Sta. (1927).

[28] Tarr, L. W., Fruit Jellies. I. The Role of Acids, Bull. 134, Delaware Agr. Exp. Sta. (1923).

[29] Tarr, L. W., and Baker, G. L., Fruit Jellies. II. The Role of Sugar, Bull. 136, Delaware Agr. Exp. Sta. (1924).

[30] Tarr, L. W., Fruit Jellies. III. Jelly Strength Measurements, Bull. 142, Delaware Agr. Exp. Sta. (1926).

[31] Myers, P. B., and Baker, G. L., Fruit Jellies. IV. The Role of Salts, Bull. 144, Delaware Agr. Exp. Sta. (1926).

[32] Myers, P. B., and Baker, G. L., Fruit Jellies. VI. The Role of Pectin. II. The Extraction of Pectin from Pectic Materials. Bull. 160, Delaware Agr. Exp. Sta. (1929).

use of approximately 0.052 per cent tartaric, and 0.066 per cent citric acid in the final gel. The quantity of pectin required depends, of course, upon its quality, as noted above. It may be as low as 0.3 and as high as 0.7 per cent.

QUANTITATIVE ESTIMATION OF PECTIC SUBSTANCES.—The commonest and simplest method of determining pectin is to precipitate the boiled and filtered solution with alcohol. The precipitate is then washed with alcohol, dried, and weighed. This eliminates most of the proteins, but not the polysaccharides, such as starch and gums.

The method devised by Carré and co-workers [33] for the estimation of pectin is the most definite of any yet proposed. The pectin is saponified with 0.1 *N* sodium hydroxide, acidified with acetic acid, and the pectic acid precipitated as the calcium salt by means of calcium chloride. The calcium pectate is very insoluble, and can be washed very free from impurities. As prepared from apple pectin it has a composition corresponding to the formula, $C_{17}H_{22}O_{16}Ca$.

Miss Carré has extended this determination to apply to all pectic substances, and has used it in extensive investigations on apples.[34] The finely pulped tissue is first extracted with cold water to remove the pectin. The residue is boiled with 0.0133 *M* hydrochloric acid for 3 to 5 periods of 3 hours each. This dissolves the pectose, which occurs as thickenings on the cell walls. The dissolved material is then saponified and converted into calcium pectate as outlined above. The residue, containing now only the pectic substance of the middle lamella, is boiled with 0.0133 *M* sodium hydroxide for several periods of ½ hour each. This dissolves the middle lamella and converts it into pectic acid, which is determined as before as calcium pectate.

Another method that has been suggested for determining the pectic substances as a whole consists in measuring the carbon dioxide produced by the decarboxylation of the uronic acids when the material is boiled with 12 per cent hydrochloric acid.[35] Although this is apparently a quantitative measure of the uronic acid content, these acids are found in other substances which may occur associated with the pectins.

[33] Emmett, A. M., and Carré, M. H., A Modification of the Calcium Pectate Method for the Estimation of Pectin, *Biochem. J.*, 20: 6–12 (1926). [*Cf. Biochem. J.*, 16: 60–69 (1922).]

[34] Carré, M. H., An Investigation of the Changes Which Occur in the Pectic Constituents of Stored Fruits, *Biochem. J.*, 16: 704–712 (1922). [*Cf. Ann. Botany*, 39: 811–839 (1925).]

[35] Dore, W. H., The Composition of Pectin: A Preliminary Report of the Determination of Galacturonic Acid in Pectin, *J. Am. Chem. Soc.*, 48: 232–236 (1926). (*Cf.* Nanji, Paton, and Ling, *loc cit.*)

ACTION OF ENZYMES ON PECTINS.—There are generally recognized three pectic enzymes,[36] (1) protopectinase, (2) pectase, and (3) pectinase.

Protopectinase hydrolyzes the middle lamella substance away from the cellulose walls, thus macerating the tissue. Presumably the product formed is pectin, although this has never been prepared from the middle lamella by enzymic process. The enzyme is demonstrated only by its macerating action on plant tissue, usually a storage tissue like tubers, fleshy roots, and fruit.

Pectase is an esterase, and hydrolyzes the methoxy groups from pectin, producing pectic acid. It is demonstrated by allowing it to act on pectin in the presence of calcium, when a gel of calcium pectate is produced.

Pectinase hydrolyzes pectin (and possibly pectic acid) to its simple components, sugars and galacturonic acid. Its action on pectin sols can be followed by the formation of these reducing substances, by the lowering of viscosity, or by the appearance of precipitated material, probably lignin. Pectinase preparations are used commercially for the destruction of pectin in fruit juices to enhance their filtrability.[37–39] Pectinase will probably be found to be a mixture of enzymes, because of the variety of linkages which occur in pectin. In fact, Ehrlich [40] uses the term "pectolase" for an enzyme specifically hydrolyzing polygalacturonic acid.

It is not known what enzyme hydrolyzes acetic acid away from pectin. Protopectinase has so far been found largely in certain parasitic fungi. Its function is probably to enable the fungus to penetrate the tissue by dissolving the middle lamella. The function of pectase is more obscure. It is found most abundantly in leaves, and to a lesser extent in certain fungi. Bertrand and Mellèvre [41] found it in a large number of plants, mostly in tissues where growth was vigorous. Pectinase is obviously a digestive enzyme secreted by fungi for making available the constituents of pectin.

The relations among the pectic substances, their interconversion by

[36] Davison, F. R., and Willaman, J. J., Biochemistry of Plant Diseases. IX. Pectic Enzymes, *Botan. Gaz.*, 83: 329–361 (1927).

[37] Kertesz, Z. I., A New Method for Enzymic Clarification of Unfermented Apple Juice, New York Agr. Exp. Sta. (Geneva) Bull. 589 (1930).

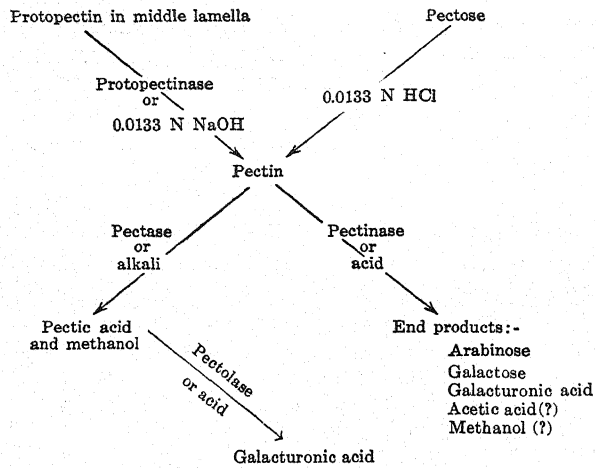
[38] Willaman, J. J., and Kertesz, Z. I., The Enzymic Clarification of Grape Juice, New York Agr. Exp. Sta. (Geneva) Tech. Bull., 178 (1931).

[39] Mehlitz, A., Verfahren zur Herstellung von Fruchtsäften, Süssmosten und dergleichen, *Konserven Ind.*, 17: 306–307, 321–322, 729–730, 742–744, 756–758 (1930).

[40] Ehrlich, F., Über die Pektolase, ein neuaufgefundenes Pektinferment, I and II, *Biochem. Z.*, 250: 525–534; 251: 204–222 (1932).

[41] Bertrand, G., and Mallèvre, A., Sur la diffusion de la pectose dans le règne végétal et sur la préparation de cette diastase, *Compt. rend.*, 121: 726–728 (1895).

means of chemicals, and the action of their enzymes are shown in the following diagram.



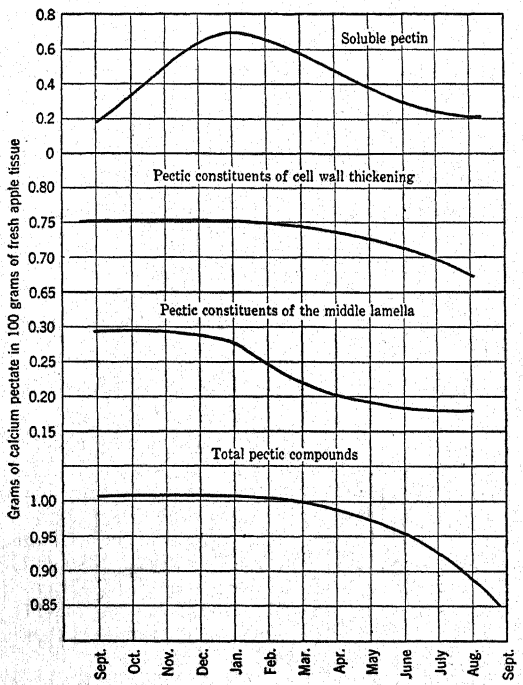


FIG. 156.—Showing the pectic changes which take place in apples from the time of picking to the last stages of senescence (Data of Carré and Horne.)

It should be pointed out that there are two steps in this scheme which have not as yet been demonstrated. One is the enzymic hydrolysis of pectose. We do not know whether protopectinase will accomplish this, or whether pectose has its own enzyme. This is on the assumption, of course, that Carré's pectose is different from the middle lamella protopectin. The other gap in our knowledge is the action of pectinase. Investigators have always used pectin for its substrate. The fate of the methanol in this case is unknown. Whether pectinase can act on pectic acid or on one of its soluble salts is not known.

Table LXXVII presents a brief summary of the occurrence of the pectic enzymes. A thorough review of these enzymes has been made by Kertesz.[42]

TABLE LXXVII

OCCURRENCE OF PECTIC ENZYMES. PARTIAL LIST

(+ = present; − = absent; ... = not tested)

Source	Protopectinase	Pectase	Pectinase
Rhizopus tritici	+	−	+
Rhizopus oryzae	+	+	+
Rhizopus nigricans	+	+	+
Aspergillus niger	−	−	+
Clostridium sp.	+	+	+
Sclerotinia libertiana	+	+	+
Sclerotinia cinerea	−	+	+
Trifolium pratense (leaves)	−	+	−
Nicotiana tabacum (leaves)	...	+	...
Takadiastase	+	−	+
Zea mays (pollen)	−	+	−
Barley malt	−	+	+
Emulsin	+	−	+
Mespilus germanica (fruit)	+	+	−
Daucus carota (root)	−	+	−
Syringa vulgaris (leaves)	...	+	...
Solanum tuberosum (leaves)	...	+	...

BIOLOGICAL SIGNIFICANCE OF PECTIN.—The pectic substance of the middle lamella apparently functions as a cementing material between cells. During the ripening of fruit this lamella is dissolved, the cells separate from each other, and the tissue disintegrates. The retting of flax has been shown to be a bacterial dissolution of the pectic material between fibers.

Carré and Horne[43] have followed in great detail the changes in the

[42] Kertesz, Z. I., Pectic Enzymes, *Ergeb. Enzymforsch.*, 5: 233–258 (1936).

[43] Carré, M. H., and Horne, A. S., An Investigation of the Behaviour of Pectic Materials in Apples and Other Plant Tissues, *Ann. Botany*, 41: 193–237 (1927).

pectic substances of apples during the after-ripening period in cold storage. They used the chemical methods outlined above for distinguishing pectin, pectose, and the middle lamella substance, and also microchemical methods based on the specific staining of pectic substances by ruthenium red $[Ru_2(OH)_2Cl_4 \cdot 7(NH_3) \cdot 3H_2O]$. The changes which they found are shown in Fig. 156.

Appleman and Conrad [44] found that during the ripening of peaches the total pectic substances remained constant, but that there was a decided decrease in protopectin and a corresponding increase in pectin. They found that in tomatoes this change is very rapid, and that the disintegration of the fruit during the canning process is the greatest when the ratio of pectin to protopectin is greatest.

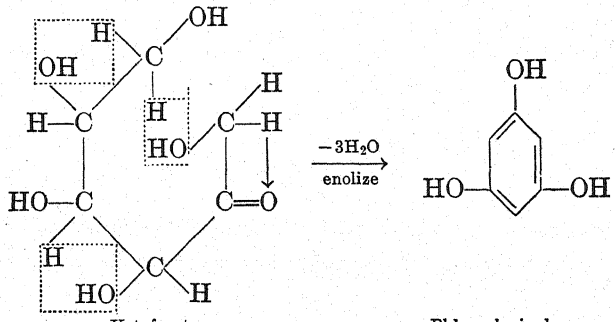
[44] Appleman, C. O., and Conrad, C. M., Pectic Constituents of Peaches and Their Relation to Softening of the Fruit, Bull. 283, Maryland Agr. Exp. Sta. (1926); The Pectic Constituents of Tomatoes and Their Relation to the Canned Product, Bull. 291, Maryland Agr. Exp. Sta. (1927).

CHAPTER XXIX

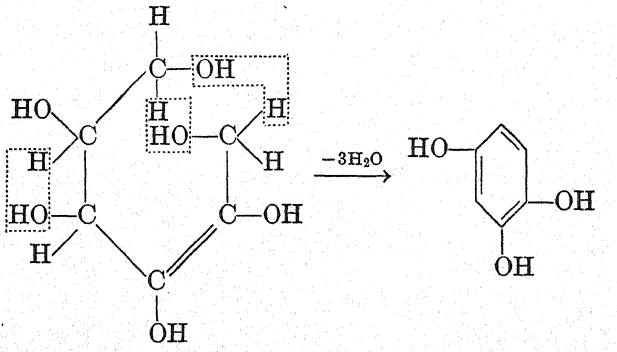
LIGNIN AND THE TANNINS

Perhaps the only justification for grouping both lignin and the tannins in one chapter is the fact that both of these series of compounds contain polyhydroxy aromatic nuclei so that at least in that respect they have certain features in common.

Presumably both lignin and the tannins are derived in some way from the carbohydrate nucleus. Wislicenus [1] suggests that the phenolic groupings characteristic of lignin (and presumably also of the tannins) may arise by the dehydration of fructose as indicated in the following diagrams where the end products of the dehydration are pyrogallol, phloroglucinol, and hydroxyhydroquinone:

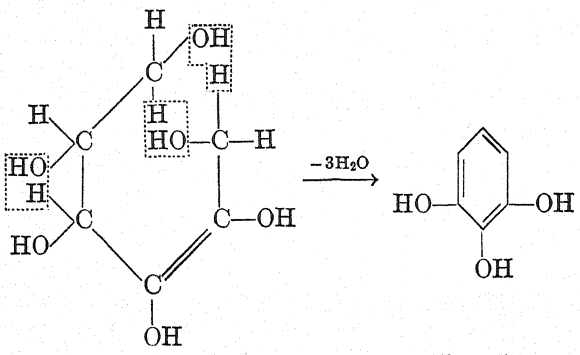


Ketofructose — Phloroglucinol



2-3-Enolfructose — Hydroxyhydroquinone

[1] Wislicenus, H., Über Ur-Cellulose und Ur-Lignin in den holzbildenden Pflanzensäften als chemische Baustoffe der Holzsubstanz, *Papier Fabrik.*, 31 (Festheft): 65–77 (July, 1933).



2-3-Enolfructose Pyrogallol

LIGNIN

In 1838, Payen treated wood with nitric acid and potassium hydroxide and, besides obtaining a more or less pure cellulose, he noted that the treatment had removed some product which was richer in carbon than the residual cellulose. This material he designated as "*matieres encrustants*," although the term *lignin* had been applied to this material in 1833 by de Candolle. Lignin is still often referred to as a cellulose-encrusting material. Since these early observations a very voluminous literature has been built up in the field of lignin chemistry,[2] and there is still considerable controversy with regard to the actual structure of the lignin molecule or molecules, for it is uncertain whether or not there may be several lignins.

THE EXTRACTION AND ESTIMATION OF LIGNIN.—The two methods which have been most generally used for the extracting and the quantitative estimation of lignin are (1) the 72 per cent sulfuric acid method, and (2) the fuming hydrochloric acid method. When a woody tissue containing lignin is treated with 72 per cent sulfuric acid under rather definite experimental conditions,[3] the cellulose and other struc-

[2] The reader is referred to the following sources for essential literature: Fuchs, W., Die Chemie des Lignins, Julius Springer, Berlin (1926); Hess, K., Die Chemie der Zellulose und ihrer Begleiter, pp. 129–199, Akademische Verlagsgesellschaft, Leipzig (1928); Hägglund, E., Holzchemie, pp. 84–153, Akademische Verlagsgesellschaft, Leipzig (1928); Phillips, M., The Chemistry of Lignin, *Chem. Rev.*, 14: 103–170 (1934), 304 references; Hibbert, H., *et al.*, Studies on Lignin and Related Compounds, I-IX, XII-XIX, *Can. J. Research*, 2: 357–363, 364–375 (1930); 3: 65–69, 130–139 (1930); 4: 110–118, 119–133, 240–253 (1931); 5: 1–47, 302–305 (1931); 13: 28–34, 35–47, 48–54, 55–60, 61–77, 78–87, 88–102, 103–113 (1935); X, *J. Am. Chem. Soc.*, 55: 4720–4727 (1933); and Norman, A. G., The Biochemistry of Cellulose, the Polyuronides, Lignin, etc., Clarendon Press, Oxford, England (1937).

[3] Ritter, G. J., Seborg, R. M., and Mitchell, R. L., Factors Affecting Quantitative Determination of Lignin by 72 Per Cent Sulfuric Acid, *Ind. Eng. Chem., Anal. Ed.*, 4: 202–204 (1932).

tural elements are dissolved or rendered water-soluble and on dilution with water the lignin remains as an insoluble precipitate which can be filtered, washed free of sulfuric acid, dried, and weighed, and thus the lignin content of the tissues can be estimated. An alternative method is the use of fuming hydrochloric acid.[4] Aqueous or alcoholic sodium hydroxide, on the contrary, dissolves the lignin and leaves the cellulose as the insoluble residue. The pulping of wood is almost wholly concerned with bringing the lignin into a soluble form, so that the cellulose fibers can be recovered essentially lignin-free, although most paper pulp contains from 1 to 5 per cent of lignin. It is generally agreed that lignin is probably changed during the process of isolation. The difficulty with solving the chemical problems of lignin chemistry lies in the fact that lignin itself is amorphous, forms colloidal sols in those media in which it is dispersible, and, until the lignin molecule is pretty thoroughly broken down, does not yield crystalline compounds which can be readily characterized.

LIGNIN DERIVATIVES.—Lignin, as isolated, contains methoxy groups, the methoxyl value ranging from 11.60 to 21.0 per cent, depending in part on the botanical source from which the lignin is derived but still more upon the method which was used for its isolation. The lowest methoxyl content is reported for spruce lignin isolated by the fuming hydrochloric acid method; the higher values are for the lignin of the sugar maple isolated by the 72 per cent sulfuric acid method. Lignin can be further alkylated up to a maximum of at least 26.79 per cent $-OCH_3$ groups by appropriate chemical methods.

Lignin, as isolated, can be acetylated with either acetic anhydride or acetyl chloride, and the literature reports acetylated lignin containing acetyl groups in amounts ranging from 19.85 to 37.85 per cent. Similarly it can be benzoylated to yield benzoyl derivatives, and various other aromatic and aliphatic acid chlorides have been used to introduce the desired groupings.

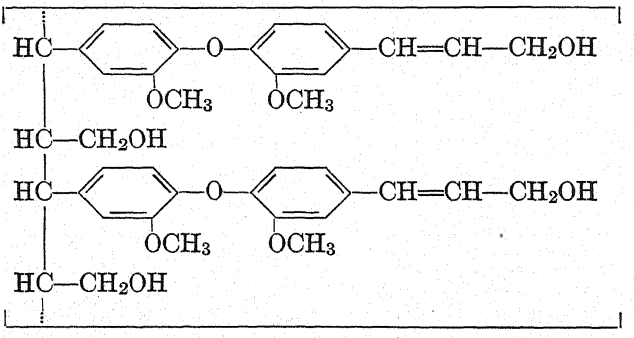
Lignin has been both halogenated and nitrated to form chlor-, brom-, or nitro-derivatives. It takes up approximately 30 per cent of chlorine or 40 per cent of bromine. Twenty-eight per cent of the bromine was "firmly bound." One empirical formula for a "nitrolignin" approximated $C_{42}H_{37}N_3O_{24}$. In the pulping of wood by the sulfite process, lignin is sulfonated to yield lignosulfonic acids. α- and β-Sulfonic acids have been described, agreeing more or less with the formula $C_{26}H_{30}SO_{12}$. In all probability, however, the compounds which have been described are mixtures of several substances.

On oxidation lignin yields a great variety of products, mostly aliphatic and aromatic acids. Among the compounds which have been reported are formic, acetic, oxalic, succinic, fumaric, malonic, adipic,

[4] Phillips, M., The Quantitative Determination of Methoxyl Lignin, and Cellulose in Plant Materials, *J. Assoc. Official Agr. Chem.*, 15: 118–131 (1932).

benzoic, phthalic, isophthalic, mellitic, hemimellitic, trimellitic, and anisic acids. Vanillin has also been obtained. On reduction with hydroiodic acid and phosphorus a mixture of liquid and solid hydrocarbons, ranging in molecular weight from 167 to 842, has been obtained. No pure hydrocarbon was identified; however, no *n*-hexyliodide was obtained, indicating that the straight C_6 carbon chain of the sugars was absent. Distillation with zinc dust has yielded guaiacol, catechol, and 1-*n*-propyl-3-methoxy-4-hydroxybenzene. Fusion with alkalies has yielded oxalic acid and other aliphatic acids, catechol, protocatechuic acid, vanillic acid, and "lignic acid," a dark, amorphous humin-like product. Dry distillation has yielded acetone, methyl alcohol, acetic acid, eugenol, 5-hydroxymethylfurfural, phenol, *o*-cresol, 1-vinyl-3-methoxy-4-hydroxybenzene, and 1-*n*-propyl-3-methoxy-4-hydroxybenzene. Dry distillation has also yielded a series of saturated and unsaturated hydrocarbons. The saturated hydrocarbons isolated have had empirical formulas $C_{13}H_{26}$, $C_{14}H_{26}$, $C_{16}H_{30}$, $C_{24}H_{44}$, and $C_{30}H_{60}$. The unsaturated hydrocarbons have had empirical formulas $C_{11}H_{16}$, $C_{12}H_{16}$, and $C_{13}H_{16}$.

THE STRUCTURE OF LIGNIN.—In the final determination of the structure of lignin all the above compounds must be taken into consideration. Nearly all the recent workers seem to agree that coniferyl alcohol or coniferyl aldehyde or some closely related compound is the essential grouping in the lignin molecule. Kurschner suggests that lignin is a polymer of the glycoside coniferin, and proposes the formula:



Segment of Kurschner's lignin

However, since no *n*-hexyliodide was isolated from the reduction products, it seems improbable that Kurschner's formula will hold.

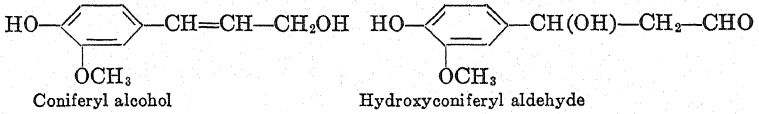
Klason [5] proposes that lignin is a single chemical compound formed by the condensation of nine molecules of oxyconiferyl alcohol (coniferyl aldehyde where a molecule of water has been added at the

[5] See numerous papers in *Berichte* and *Svensk Pappersförädlingstidskrift* since 1920.

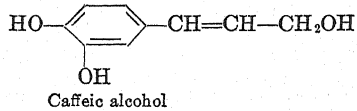
double bond) joined by the ether linkage from the phenol group of one coniferyl aldehyde unit to the aldehyde group of the next unit, with the two end methoxy residues replaced by acetyl groups.

This is essentially the picture that Freudenberg *et al.*[6] have adopted. Freudenberg notes that "lignin, like cellulose, is characterized by the regular recurrence of a definite structural unit which in this case is probably caffeic alcohol."

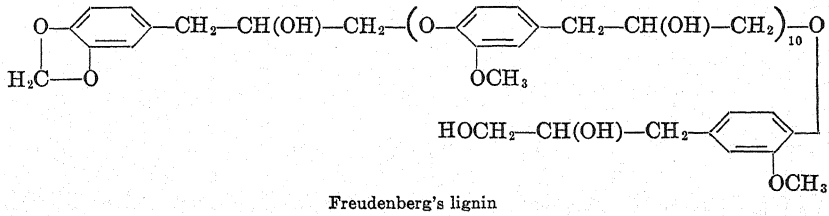
He suggests that 12 molecules of caffeic alcohol condense with themselves to form a "primary lignin" having the formula $C_{120}H_{144}O_{37}$, with the structure indicated.



Coniferyl alcohol Hydroxyconiferyl aldehyde

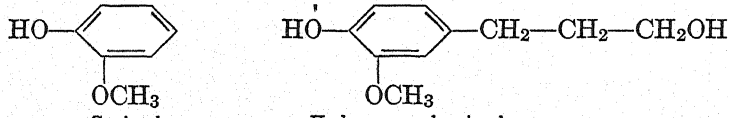


Caffeic alcohol



Freudenberg's lignin

He notes that in the formula which he gives the linkages are depicted as taking place in the *para*-position, although a part of the units may be linked *meta* instead of *para*. Phillips[7] suggests that the fundamental units are guaiacol and *n*-hydroxypropylguaiacol, "where the hydrogens in the *n*-propyl side-chain are substituted by alcohol hydroxyl groups." Fuchs[8] gives a somewhat different and much more



Guaiacol *n*-Hydroxypropylguaiacol

[6] Freudenberg, K., Sohns, F., Dürr, W., and Niemann, C., Über Lignin, Coniferylalkohol und Saligenin, *Cellulosechem.*, 12: 263–276 (1931); Freudenberg, K., The Relation of Cellulose to Lignin in Wood, *J. Chem. Education*, 9: 1171–1180 (1932); and Die Beziehung der Cellulose zum Lignin im Holze, *Der Papier-Fabrik.*, 30: 189–197 (1932).

[7] Phillips, M., The Isolation of Normal Propyl Guaiacol as a Degradation Product of Lignin, *Science*, 73: 568–570 (1931).

[8] Fuchs, W., Untersuchungen über Lignin, Huminsäuren und Humine, *Z. angew. Chem.*, 44: 111–118 (1931).

complicated formula, but the weight of evidence appears to be in favor of something approximating either Klason's or Freudenberg's configuration.

Ehrlich [9] does not give a structural formula for lignin but suggests that pectin is the precursor of lignin. He notes that lignified tissues contain cellulose and hemicelluloses but little or no pectin and that non-lignified tissues contain relatively little hemicelluloses but large amounts of pectin. He was able to secure a fraction of pectin soluble in 70 per cent alcohol that contained 11.6 per cent of methoxy groups and that had a carbon and hydrogen percentage more or less identical with that of lignin, and he proposes as a working hypothesis the equation

$$\underset{\text{Flax pectic acid}}{C_{46}H_{68}O_{40}} \rightarrow \underset{\text{Flax lignin}}{C_{45}H_{48}O_{16}} + CO_2 + 10H_2O + 6O_2$$

Hibbert gives the empirical formula of " native lignin " as

$C_{42}H_{32}O_6(OCH_3)_5(OH)_5$, and alkali lignin-A as
$C_{61}H_{46}O_{10}(OCH_3)_6(OH)_7$, and alkali lignin-B as
$C_{99}H_{69}O_{17}(OCH_3)_9(OH)_{11}$.

THE FUNCTION OF LIGNIN.—The function of lignin in the plant is apparently to give strength and rigidity to the cell wall. The unlignified tissue is usually soft, contains a higher percentage of water, and has a much lower breaking strength. Lignin is extremely resistant to decay and bacterial action, for woods which have been buried for at least 500,000 years still contain the great bulk of the original lignin in an apparently unaltered state.[10] We have already referred to the fact that Payen called lignin an encrusting material. For many years there has been an extremely lively controversy as to whether or not lignin is simply an encrustant surrounding the cellulose fibers and cementing them together or whether the lignin is actually combined with the cellulose to form lignocelluloses. Both views have been vociferously supported by different workers. As is usual where both sides have such ardent supporters, it now [11] appears as if both views were correct. In aspen wood no evidence could be found for the presence of a lignocellulose, and all the lignin in aspen appears to be present as an encrusting material. In Jack pine wood, on the other hand, approximately two-thirds of the lignin behaves exactly as does all the

[9] Ehrlich, F., Über die Chemie des Pektins und seine Beziehungen zur Bildung der Inkrusten der Cellulose, *Cellulosechem.*, 11: 161–170 (1930).

[10] Gortner, W. A., The Analysis of Glacial and Preglacial Woods, undergraduate thesis, University of Minnesota (1934).

[11] Aronovsky, S. I., and Gortner, R. A., The Cooking Process, IX. Pulping Wood with Alcohols and Other Organic Reagents, *Ind. Eng. Chem.*, 28: 1270–1276 (1936); X. Pulping Wood with Alcoholic Nitric Acid Solutions, *ibid.*, 29: 1431–1434 (1937).

lignin in aspen, *i.e.*, it is an encrustant. Approximately one-third, however, is much more difficult of removal and appears to be combined with the cellulose as a lignocellulose. Therefore, the question of the presence or absence of a lignocellulose is probably dependent on the botanical species of lignified tissue which is under investigation.

LIGNIN IN ANIMAL METABOLISM.—We have already indicated that lignin is extremely resistant to decay and bacterial action, and it seems probable that it contributes relatively little, if at all, to the energy requirements of animals. When it is fed to dogs or cows,[12] there is a partial breakdown of the lignin in the animal body as evidenced by an increase in the amount of benzoic acid eliminated as hippuric acid and also in a loss in methoxy content of the lignin as re-isolated from the feces. Csonka *et al.* conclude that such breakdown of lignin as does occur is probably not due to bacterial action but that it takes place in the stomach, possibly through some enzyme present in the gastric juice.

THE TANNINS

Tannin is a generic name for a group of substances widely distributed in the higher plants and showing certain characteristic physical and chemical properties. Tannins may be found in leaves, tea (15 per cent), sumac, *Rhus coriaria* (13–15 per cent), rhododendron, etc. They occur also in wood and stems, and especially in the bark of the oaks, hemlock, etc.; in fruits, especially unripe fruits, *e.g.*, persimmons, plums, hulls of walnuts and of butternuts and hickory nuts; in seeds (more rarely), but especially in the seed coats; in algae, fungi, and pathological plant growths. Plant galls may contain as much as 75 per cent tannin and rarely contain less than 25 per cent.

Only one instance is recorded of a tannin from an animal source. Three per cent of a substance was extracted from corn weevils which, on hydrolysis with dilute acids, yielded dextrose, gallic acid, and a red "phlobaphene." The existence of a true animal tannin may well be doubted, for this product may have been derived from undigested food remains.

Tannins may be characterized by the following properties:

1. They are, in general, amorphous, rarely crystalline.
2. They have an astringent taste.
3. They give colors (inks) with ferric salts.
4. They are precipitated from solution by $K_2Cr_2O_7$ and by alkaloids.
5. They precipitate gelatin from solution, which property enables them to convert hide into leather.

[12] Csonka, F. A., Phillips, M., and Jones, D. Breese, Studies on Lignin Metabolism, *J. Biol. Chem.*, 85: 65–75 (1929).

6. Their sols develop a deep red color on the addition of potassium ferricyanide.

7. They are readily soluble in hot water to form solutions which are in reality colloidal sols.

8. They all contain polyhydroxyphenols or derivatives of polyhydroxyphenols, in many instances in complex condensed ring structures. A part of the tannins are hydrolyzable with acids to yield phenolic derivatives and in some instances sugars, usually *d*-glucose. Sugars, however, are not an essential part of the tannin molecule, and when the sugar is removed by appropriate means, the residue which remains still shows the properties characteristic of tannins as a class.

9. They may act as a chromogen for oxidases, *e.g.*, the green walnut hulls when broken open darken at once, as the result of oxidation of tannin by oxidases or even by exposure to air.

10. When tannins are heated with dilute acids, insoluble amorphous anhydrides or "*phlobaphenes*" (apparently anhydrides of the tannin) are produced besides other hydrolysis products. These phlobaphenes are produced by any process tending to cause the tannin to lose water. They are red or brown substances, practically insoluble in water, chemically relatively inert, and occur in nature only associated with the tannins.

Various classifications have been proposed for the tannins. The one noted below was originally proposed by Freudenberg [13] and has been used by Nierenstein.[14]

FREUDENBERG'S CLASSIFICATION OF THE TANNINS

I. Condensed tannins
  A. Catechin tannins
    Acacatechin tannins
    Isoacacatechin tannins
    Gambir catechin tannins
  B. Maclurin tannins
II. Hydrolyzable tannins
  A. Gallotannins
  B. Ellagitannins
  C. Caffetannins
III. Unclassified tannins

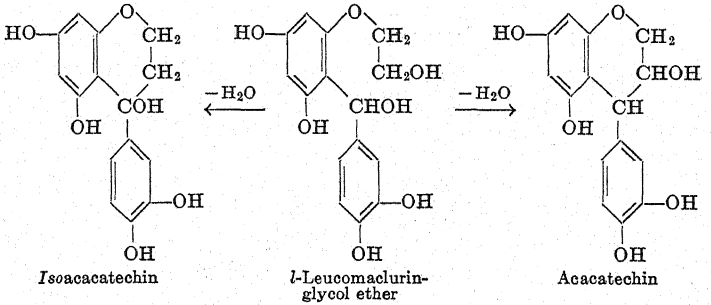
THE CONDENSED TANNINS.—The condensed tannins all contain the phloroglucinol nucleus. They cannot be hydrolyzed by acids or enzymes and apparently represent polymers of relatively simple poly-

[13] Freudenberg, K., Die Chemie der natürlichen Gerbstoffe, Berlin (1920).

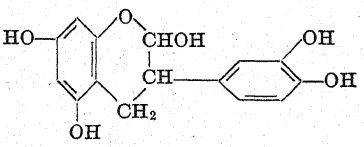
[14] Nierenstein, M., The Natural Organic Tannins, The Sherwood Press, Cleveland, Ohio (1935).

hydroxy compounds containing aromatic nuclei. The chief tannins of commerce which fall in this class are Indian cutch and cube gambir. Indian cutch is the tannin extracted from *Acacia catechu*, *A. catechuoides*, and *A. sundra*. Cube gambir is the tannin in the extract from *Uncaria gambir*, *U. acida*, *U. dasyoneura*, *U. Bernaysii*, and *U. lanosa*. Indian cutch and cube gambir have been articles of commerce and used in medicine since the dawn of European history. They were introduced into Europe from the Far East and are referred to by Pliny in his *Historia Naturalis*. However, only within the last twenty years have the structural problems presented by these tannins been elucidated by the brilliant researches of Karl Freudenberg and M. Nierenstein. Indian cutch is the condensation product of *acacatechin* with some *isoacacatechin*, whereas gambir contains the *d-catechin* nucleus.

It will be noted that, in the formulas of the various catechins, there are two asymmetric carbon atoms. Consequently there is a series of fifteen total possible stereoisomers. Nierenstein notes that eight of these isomers have been identified as occurring in nature. Nierenstein observed that in *Acacia catechu* the catechin occurs in the heartwood, so he concluded that it must be a final product of plant metabolism. In the young twigs he discovered *l*-leucomacluringlycol ether, and he shows how this compound by the loss of water condenses to form acacatechin and *iso*acacatechin.



*Iso*acacatechin — *l*-Leucomacluringlycol ether — Acacatechin

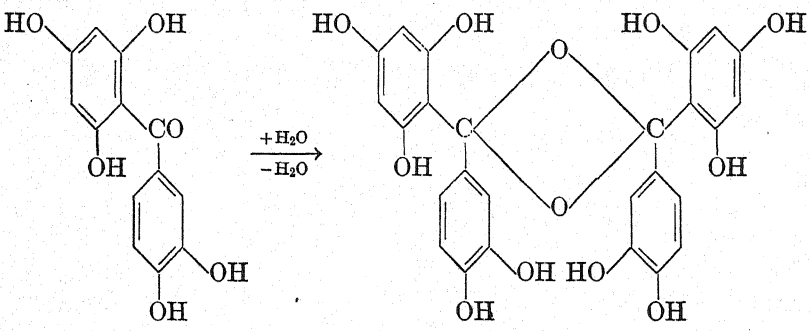


Gambir catechin

Although Indian cutch and cube gambir are the principal catechin tannins of commerce, catechin tannins also occur in rhubarb, the cacao bean, the cola nut, in mahogany wood, and in a considerable number of the species of eucalyptus, as well as in a number of other less

common plant families. In certain of the tannins of this group the catechin residue is present in the form of a carboxylic acid which allows for its condensation with the phenolic group of another catechin residue. In some of the tannins, glucose is attached by a glycosidal linkage to one of the hydroxyl groups.

The maclurin tannins are apparently condensation products of the maclurin nucleus. Nierenstein suggests that the mori-tannin which occurs in the wood of *Chlorophora tinctoria* is the condensation product of two molecules of maclurin brought about by first the addition and then the subtraction of two molecules of water.



Maclurin

Mori-tannin (Nierenstein)

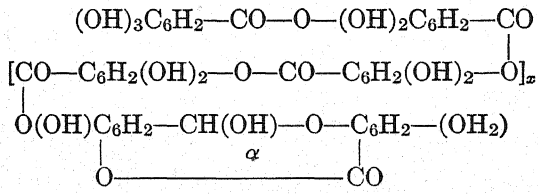
THE HYDROLYZABLE TANNINS.—These may be divided into the gallotannins, the ellagitannins, and the caffetannins, yielding respectively gallic acid or *m*-digallic acid, ellagic acid, or caffeic acid and quinic acid.

The gallotannins are very widely distributed in plants, and the "tannin" of commerce is one of their decomposition products, gallotannic acid or digallic acid. This is an amorphous powder, soluble in water, acid to litmus, soluble in alcohol and glycerol, but only very slightly soluble in other organic solvents, such as ether, benzene, and carbon bisulfide. It possesses a very astringent taste. The chief commercial source of the gallotannins is Chinese nut galls. Fischer, in his studies on the tannins, came to the conclusion that they were glucose derivatives in which one molecule of glucose was combined with ten molecules of gallic acid. He accordingly concluded that Chinese nut gall tannin corresponded to a pentadigalloyl glucose where one molecule of digallic acid is esterified on each of the five hydroxyl groups of the glucose molecule in the same way that glucose forms a pentaacetyl derivative. Fischer and Freudenberg[15] synthesized a

[15] Fischer, E., and Freudenberg, K., Über das Tannin und die Synthese ähnlicher Stoffe, I., *Ber.*, 45: 915–935 (1912); III, *Ber.*, 46: 1116–1138 (1913).

pentagalloyl glucose and considered that it was in all probability closely related to, if not identical with, the tannin of the Chinese nut gall. In the course of their work they prepared hepta(tribenzoylgalloyl)-*p*-iodophenylmaltosazone, $C_{220}H_{142}O_{58}N_4I_2$, which has a molecular weight of 4,021. This compound has one of the highest molecular weights of any compound which has so far been synthesized and the structure of which is definitely known. The iodine was deliberately introduced into the molecule in order to provide for an accurate analysis, since in such a large molecule the analysis for carbon and hydrogen alone would not be sufficiently accurate to determine structural relationships.

Later, however, Nierenstein showed that Fischer's idea of the natural tannin being related to a pentadigalloyl glucose was incorrect, inasmuch as he demonstrated that only the α-glycoside linkage of glucose was united to the gallic acid residue, and he was able to hydrolyze off the glucose and still retain the characteristic properties of the tannin. Nierenstein [16] believes that the nut gall is a polydigalloylleucodigallic acid anhydride which may sometimes occur in the form of an α-glycoside where the glucose residue is attached to the —OH group designated by α.



Nierenstein's gallotannin

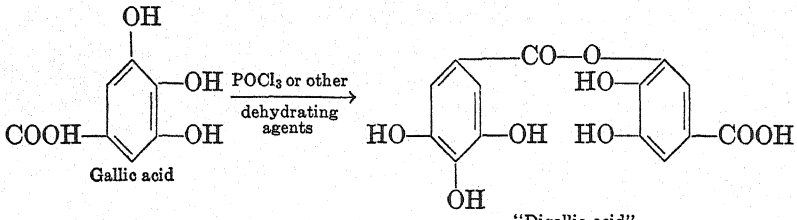
The tannins of *Acer ginuala* and of Chinese rhubarb are apparently much simpler compounds than the tannin of nut galls. The acertannin seems to be digalloylaceritol where the carboxyl group of gallic acid is esterified to hydroxyl groups of the aceritol. The tannin of Chinese rhubarb appears to be simply gallic acid-β-glucoside.

The tannin [17] of witch hazel, *Hamamelis virginica*, is apparently the digalloyl derivative of the very unusual hexose, hamamelose,[18] where the gallic acid is combined with the two primary alcohol groups of the sugar.

[16] Nierenstein, M., Spiers, C. W., and Hadley, A. C., Gallotannin. XIV. The Action of Yeast on Gallotannin, *J. Am. Chem. Soc.*, 47: 1726–1728 (1925).

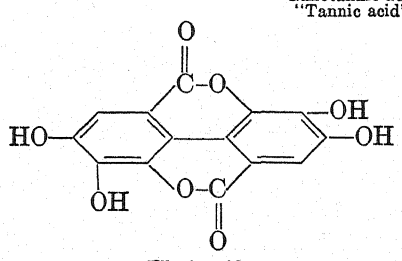
[17] Freudenberg, K., and Blümmel, F., Hamameli-tannin. III., *Ann.*, 440: 45–59 (1924).

[18] Schmidt, Otto Th., Die Konstitution des Zuckers aus Hamameli-tannin. Über Zucker mit verzweigter Kohlenstoffkette. I., *Ann.*, 476: 250–271 (1929).

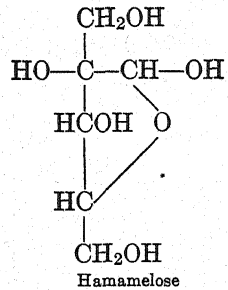


Gallic acid

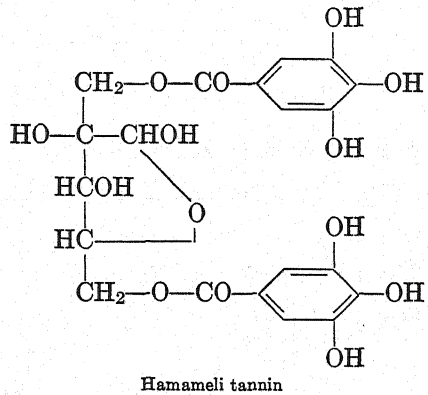
"Digallic acid"
"Gallotannic acid"
"Tannic acid"



Ellagic acid
(The dilactone of the diphenyl derivative corresponding to gallic acid)



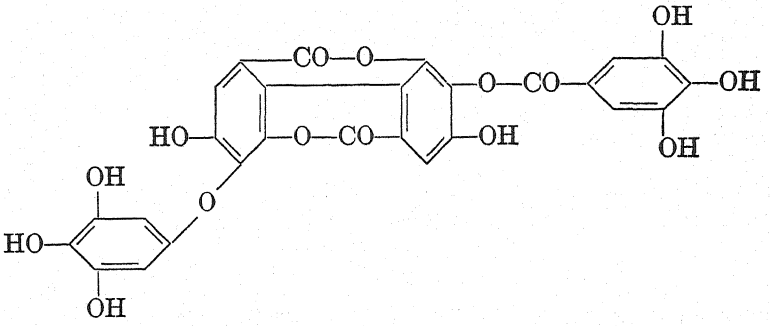
Hamamelose



Hamameli tannin

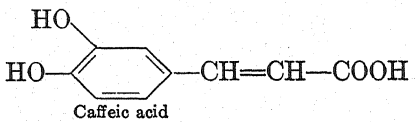
The ellagitannins are derivatives of ellagic acid, and Nierenstein suggests that they may be in many instances the mono-, di-, tri-, or tetragalloyl derivatives of ellagic acid.

These tannins include the tannin of chestnut wood, which is apparently a monogalloylellagic acid with a quercetin residue linked to one of the remaining hydroxyl groups of ellagic acid. Dividivitannin (pods of *Caesalpinia coriaria*) is apparently similar to the tannin of chestnut wood except that a $C_{14}H_{14}O_{11}$ residue replaces the $C_{15}H_9O_6$ residue of quercetin.

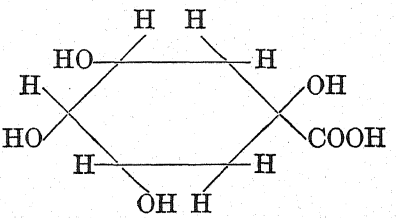


Digalloyl ellagic acid

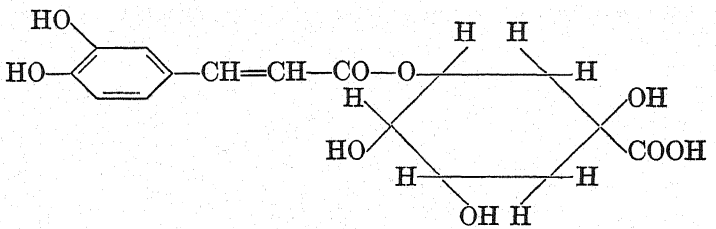
The caffetannins yield on hydrolysis caffeic acid and quinic acid and usually a residue of unidentified constitution. Possibly in most instances the caffeic acid and quinic acid are united to form chlorogenic acid, and Nierenstein suggests that the caffetannins are probably condensation products of chlorogenic acid.



Caffeic acid



Quinic acid



Chlorogenic acid

The caffetannins occur in a great variety of plants, including such important species as *Pinus laricio*, *Larix europaea*, *Coffea arabica*, *Papaver somniferum*, *Digitalis purpurea*, *Nicotiana tabacum*, etc.

Aside from the varieties of tannins noted, Nierenstein observes that there are a great number of tannins, the investigation of which has not yielded sufficient information to provide for even a hypothesis as to the structural elements which constitute the tannin. He suggests that in this group of the "unclassified tannins" there are probably

many which will fall in line with some one of the groups noted above, and that perhaps other groups of tannins will have to be formed when sufficient investigations of the various tannins have been conducted. In this unclassified group are the oak tannins, the larch tannins, the fir tannins, the sequoia tannins, the spruce tannins, the willow-bark tannins, the rhododendron tannins, etc.

THE TANNING PROCESS.—The process of tanning may be regarded as the conversion of a relatively hydrophilic colloid gel into a relatively non-hydrophilic gel which we call "leather." The manufacture of leather through the action of tannins upon animal hide is an art which dates from the earliest antiquity. It was developed exclusively as an art, and only in recent years has chemical control come into the larger tanneries. It is only natural that such a basic industry should have built up a relatively enormous literature. Wilson [19] has covered the pertinent literature to the date of his monograph. Wilson and Thomas [20] regard the tanning process as a stoichiometrical combination of the tannin with the hide substance. However, this is not the view of Gustavson,[21] McLaughlin, *et al.*,[22] nor apparently of Dorothy Jordan Lloyd,[23] for the tanning process may be completely reversed by shifting the hydrogen-ion concentration, whereby the tannin (or chrome in chrome tanning) can be completely removed with the regeneration of the hide substance which can be retanned. McLaughlin points out that in the chrome tanning process the taking up of chrome by the hide substance follows the typical Freundlich adsorption isotherm and that, when the logarithms of the chrome oxide taken up by the hide substance are plotted against the logarithms of the chrome oxide remaining in solution, a straight line is obtained, as one would anticipate from an adsorption reaction. Gustavson notes that the

[19] Wilson, J. A., The Chemistry of Leather, Vols. I and II, Second Edition, Chemical Catalog Company, New York (1928–1929).

[20] Thomas, A. W., and Foster, S. B., The Electrical Charge of Vegetable Tannin Particles, *Ind. Eng. Chem.*, 15: 707–708 (1923); Thomas, A. W., and Frieden, A., The Gelatin-Tannin Reaction, *ibid.*, 15: 839–841 (1923); Thomas, A. W., and Kelly, M. W., Concentration Factor in the Combination of Tannin with Hide Substance, *ibid.*, 15:928 (1923); The Influence of Hydrogen-Ion Concentration in the Fixation of Vegetable Tannins by Hide Substance, *ibid.*, 15: 1148–1153 (1923); The Influence of Neutral Salts upon the Fixation of Tannin by Hide Substance, *ibid.*, 15: 1262–1263 (1923); Tannic Acid Tannage, *ibid.*, 16: 800–802 (1924); Vegetable Tanning, *ibid.*, 17: 41–43 (1925).

[21] Gustavson, K. H., A Maximum Reactivity of the Hide Protein in Its Iso-Electric Zone, *J. Am. Chem. Soc.*, 48: 2963–2971 (1926).

[22] McLaughlin, G. D., Cameron, D. H., and Adams, R. S., On the Nature of the Reaction between Hide Substance and Basic Chrome Solutions, *J. Am. Leather Chem. Assoc.*, 29: 657–678 (1934).

[23] Lloyd, Dorothy Jordan, The Theory of the Tanning Process with Particular Reference to Vegetable Tanning and Chrome Tanning, *Proc. Intern. Congr. Pure and Applied Chem.*, Madrid (1934), separate, 13 pp.

maximum amount of chrome is combined with the collagen when the collagen is isoelectric. Miss Lloyd stresses in particular the water relationships and points out that the relatively non-hydrophilic tannin molecule replaces water molecules associated with the hydrated collagen and that this process of dehydration is the essential stage in the tanning process. She adds that the association between the collagen and the tannin is probably that of a covalency, for the tannin can be stripped out of the tanned skin by alkaline solutions. It appears, therefore, as though colloidal adsorption and perhaps a coacervation may account for the tanning process, since tanning proceeds most rapidly at a low *p*H where the collagen would be positively charged and the micelles of the tannin would possess a negative charge.

The technic employed for the evaluation of a tannin extract gives a definition of tannin from a practical viewpoint, *i.e.*, that portion of the water-soluble matter of certain vegetable materials which will precipitate gelatin from solution and which will combine with hide fibers to form compounds which are resistant to washing. The remaining portion of water-soluble materials is called non-tannins. The tannin-containing "solution" is shaken with purified hide powder until all the tannin has been removed from solution. This point is ascertained by testing filtered portions with gelatin to see whether a precipitate still forms.

SYNTHETIC "TANNINS."—Several substitutes for tannin in leather manufacture have been suggested. These have been called synthetic tannins, *i.e.*, substances which tan hides but which may or may not have a chemical structure analogous to natural tannins. Stiasny [24] has prepared such a "synthetic tannin" from a phenol treated with sulfuric acid and formaldehyde in the proportion of one molecule of formaldehyde to two molecules of phenol. The method of preparation suggests "Bakelite" or "Redmanol," but this substance is water-soluble. It is claimed to make a good leather and to require a shorter tanning period than is necessary when the natural tannins are used.

Meunier and Seyewetz (1908) used quinone. They found that, when gelatin was treated with phenols under conditions suitable for oxidation, the resulting precipitate became insoluble in boiling water. They state that "quinonated gelatin constitutes the most stable form of insoluble gelatin thus far known," since it resists not only boiling water but also dilute acids and alkalies. Only 2 hours at 15° C. are required to render the gelatin completely insoluble. Thomas and

[24] Stiasny, E., A Synthetic Tannin, *Leather World*, 12: 227 (1913); Syntans, New Artificial Tanning Materials, *J. Soc. Chem. Ind.*, 32: 775–777 (1913); *cf.* also Diedorf, H., Neradol D and Its Practical Applications, *J. Am. Leather Chem. Assoc.*, 8: 394–404 (1913); and Nihoul, E., The Synthetic Tannins and Their Use in Tanning, *Sci. Am. Monthly*, 1: 326–330 (1920).

Kelly [25] note that the optimum conditions for quinone tanning require an alkaline solution, approximately *p*H 8–10.

PHYSIOLOGICAL SIGNIFICANCE OF THE TANNINS.—Plant physiologists differ widely in their views as to the function of tannins in plants. Pfeffer believed that they served some useful purpose and were not merely by-products. He did not consider that they were necessary, however, to furnish benzene rings from which other compounds, such as proteins, etc., might be synthesized. Neither did he consider them as essential for cell activity. Moore suggests that the plant builds up tannins as a means of neutralizing acids by esterification. Kraus found tannins to be a direct synthetic product formed only in sunlight and carbon dioxide supply, and translocated to stems, bark, and roots. Sachs concluded that tannins occur (are produced) in the regions of most intense metabolic activity, such as in active leaves, in rapid tissue formation, such as galls, and in other pathological growths, and as a consequence of some particular stimulation. Drabble and Nierenstein conclude that cork formation is associated with tannins and that in the plant cells the complex phenols are acted upon by acids and formaldehyde, so that they are precipitated in the "cork" cell.

Other workers believe that cell wall formation or spore formation is associated, to a greater or less extent, with tannins. It has even been suggested that tannins are a special protection of the plant against animals! Cook and Taubenhaus [26] suggest that they act as an antiseptic in case of wound formation, especially as a protection against fungi. They point out that fungi are quite sensitive to tannins and that apparently the parasitic fungi are more sensitive than the saprophytic forms. It is true that tannin collects in wounded tissue in abnormally large amounts.

In green fruits tannin is more or less abundant but apparently disappears as the fruit ripens. It may not actually disappear but instead be "locked up" in giant cells in a more or less insoluble form. This seems to be the mechanism in the persimmon.[27] The Japanese "process" the persimmons by placing the unripe fruit in casks from which their rice wine (sake) has just been drawn. In five to fifteen days the casks are opened and the astringent taste will have wholly

[25] Thomas, A. W., and Kelly, M. W., Quinone Tannage, *Ind. Eng. Chem.*, 16: 925–926 (1924).

[26] Cook, M. T., and Taubenhaus, J. J., The Relation of Parasitic Fungi to the Contents of the Cells of the Host Plants. I. The Toxicity of Tannin, Bull. 91; II. The Toxicity of Vegetable Acids and the Oxidizing Enzyme, Bull. 97, Delaware Agr. Exp. Sta. (1911–1912).

[27] Gore, H. C., Experiments on the Processing of Persimmons to Render Them Nonastringent, Bull. 141, Bur. of Chem., U. S. Dept. of Agr. (1911); Large Scale Experiments on the Processing of Japanese Persimmons, Bull. 155, Bur. of Chem., U. S. Dept. of Agr. (1912).

disappeared. Gore used other anesthetics, such as ether or chloroform, to produce the same result. It was found that the tannin was localized in giant cells, some large enough to be seen with the naked eye. In the partly ripe persimmon, these cells do not break as soon as taken into the mouth but swell and eventually burst, emptying their thick tannin-bearing contents on the tongue. In the *processed* or *ripe* persimmons the contents of these cells have undergone a change, becoming more refractive and hard and losing their imbibitional power, so that they swell but slightly and do not burst. The loss of astringency presumably is due to the hardening of the contents of these giant cells.

CHAPTER XXX

CHLOROPHYLL AND THE CAROTENOIDS

Chlorophyll is the green coloring matter of plants. It occurs in the chloroplasts and is invariably accompanied by two yellow pigments, β-carotene, $C_{40}H_{56}$, and xanthophyll (lutein), $C_{40}H_{54}(OH)_2$. In some instances other carotenoids may be present in the green leaves, *e.g.*, traces of α-carotene and perhaps zeaxanthin, $C_{40}H_{54}(OH)_2$, and cryptoxanthin, $C_{40}H_{55}OH$.

Chemists from the time of Berzelius (1839) have struggled with the chemistry of chlorophyll. Willstätter[1] made the first great advance in the determination of the structure of chlorophyll. During the past ten years, Conant, Hans Fischer, Stoll, and Inman, to mention only a few of the workers, have advanced our knowledge of the structure of chlorophyll, and the actual synthesis of the chlorophyll molecule appears imminent.

Chlorophyll probably exists in the colloidal state in plants, or at least adsorbed upon colloids. It can be extracted with certain organic solvents. The earlier workers thought that various plants were characterized by different varieties of chlorophyll. Willstätter showed, however, that there is only one variety. This exists, at least as isolated in the laboratory, in two forms which have been designated chlorophyll-*a* and chlorophyll-*b*.

Chlorophyll, possessing the same properties, may be prepared from either fresh or dried leaves. One kilo of fresh leaves gives a yield of 0.9 to 2.1 grams; dried leaves yield 5 to 10 grams. The most suitable solvent for extraction is acetone (80 per cent) for dried leaves, and pure acetone for fresh leaves, sufficient acetone being added so that, allowing for the moisture in the fresh leaves, the resulting solution is 80 per cent acetone. Chlorophyll can be now isolated as readily as can any alkaloid or any sugar, and within a few hours a kilo of dried leaves should yield about 6.5 grams of practically pure chlorophyll.[2]

[1] Willstätter, R., and Stoll, A., Untersuchungen über Chlorophyll, Julius Springer, Berlin (1913); Willstätter, R., Chlorophyll, *J. Am. Chem. Soc.*, 37: 323–345 (1915); *cf.* also West, C. J., A Review of Willstätter's Researches on Chlorophyll, *Biochem. Bull.*, 3: 229–258 (1914).

[2] Schertz, F. M., The Extraction and Separation of Chlorophyll ($\alpha + \beta$), Carotin and Xanthophyll in Fresh Green Leaves, Preliminary to Their Quantitative Determination, *Plant Physiol.*, 3: 211–216 (1928); The Quantitative Determination of Chlorophyll, *ibid.*, 3: 323–334 (1928); and The Preparation of Chlorophyll, *ibid.*, 3: 487–497 (1928).

Chlorophyll is a bluish-black substance with a strong metallic luster, powdering to a greenish- or bluish-black powder. It has no definite melting point, ranging from 93° to 106° C. for various samples, and is soluble in absolute alcohol to a blue-green solution. It shows neither acidic nor basic properties. Acids change its color to olive brown and split off magnesium which is associated with the molecule.

Pure chlorophyll shows the following characteristics:

1. The ash content is 4.5 per cent, the ash being pure magnesium oxide.
2. One-third of the molecule is accounted for in the form of a monoatomic alcohol, phytol, $C_{20}H_{39}OH$.
3. The associated yellow pigments are not a part of the chlorophyll molecule.
4. When chlorophyll is saponified with potassium hydroxide in methyl alcohol, the color changes to a pure brown; impure mixtures give a dirty brown.
5. Chlorophyll is decomposed by boiling alcoholic potassium hydroxide, and there is formed a normal mixture of "phytochlorine-*e*" and "phytorhodin-*g*."
6. The absorption spectrum of solutions of pure chlorophyll is that of the fresh leaf extract. Chlorophyll-*a* shows definite absorption bands at 663, 623, 607, 577, 534, 507, 494, and 423 mμ. Chlorophyll-*b* shows absorption bands at 644, 614, 597, 567, 542, 503, 456, and 428 mμ.
7. Both chlorophyll-*a* and chlorophyll-*b* are optically active. Chlorophyll-*a* shows an optical rotation of $[\alpha]_{720}^{25} = -262°$; chlorophyll-*b*, $[\alpha]_{720}^{25} = -267°$.

Stokes, in 1864, first noticed spectrographically that chlorophyll was a mixture of two components which he called *a* and *b*. He attempted to separate them by means of a differential solubility in alcohol and carbon bisulfide. Willstätter used methyl alcohol and petroleum ether; chlorophyll-*a* goes into the petroleum ether and chlorophyll-*b* into the methyl alcohol.

Chlorophyll-*a* gives a pure yellow phase in a methyl alcoholic solution of potassium hydroxide, and gives only "phytochlorine-*e*" as a decomposition product. Chlorophyll-*a* crystallizes in thin lance-like leaflets with a blue-steel luster, m.p. 117°–120°. The solutions in ethyl alcohol are bluish-green with a deep red fluorescence.

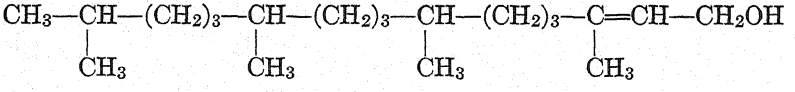
Chlorophyll-*b* gives a dark red phase with a methyl alcohol solution of potassium hydroxide and "phytorhodin-*g*" as a decomposition product. The alcoholic solution has a yellow tinge as compared with chlorophyll-*a*. Chlorophyll-*b* is completely insoluble in cold petroleum ether.

In general, the ratio of occurrence is about 1 molecule of chlorophyll-*b* to 3 molecules of chlorophyll-*a*. Willstätter determined the empirical formula of chlorophyll-*a* as $C_{55}H_{72}O_5N_4Mg + \frac{1}{2}H_2O$, and for chlorophyll-*b* as $C_{55}H_{70}O_6N_4Mg$. These empirical formulas have stood the test of time and agree with our present knowledge of the molecular structure..

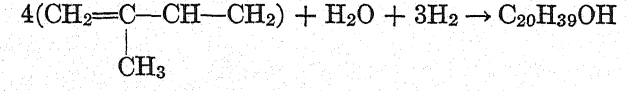
Chlorophyll may be obtained in both amorphous and in crystalline form. The Russian botanist, Borodin, discovered crystalline chlorophyll in 1881. Willstätter was unable to isolate crystalline chlorophyll in experiments dealing with approximately 200 varieties of plants. He believes that it does not occur in plants but that in the process of isolation the amorphous chlorophyll loses its phytol by action of the enzyme *chlorophyllase* (which is more or less active in alcoholic media), and the chlorophyll becomes esterified instead, with the alcohol which was used as a solvent, thus causing alcoholysis of the amorphous chlorophyll, and this derived chlorophyll product is the crystalline variety, *i.e.*, amorphous chlorophyll is phytolchlorophyllide and crystalline chlorophyll is methyl- or ethylchlorophyllide.

It is impracticable to discuss in detail the many decomposition products of the chlorophylls which have been isolated and identified. There are, however, certain of the groupings which have either been synthesized or whose structure is adequately known, and it will suffice to indicate the structural relationships of these compounds.

Phytol, which is associated with the chlorophyll nucleus, is an unsaturated aliphatic alcohol. It has been synthesized by Fischer and Löwenberg,[3] who have shown that it is 3-7-11-15-tetramethyl-Δ_{23}-hexadecan-1-ol.



Earlier workers suggested that phytol might be related to isoprene, the hydrocarbon of rubber and terpenes, four molecules of isoprene condensing with one molecule of water, followed by a reduction:

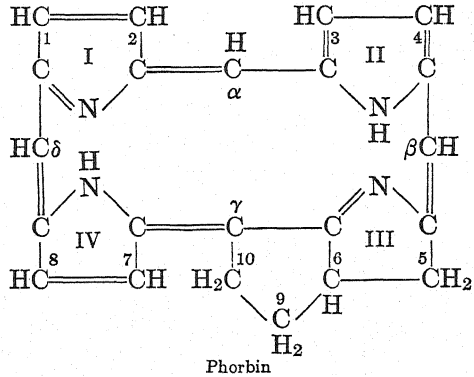


Phytol is readily absorbed[4] from the intestinal tract by the white rat, but its fate in the animal organism is unknown.

[3] Fischer, F. G., and Löwenberg, K., Die Konstitution des Phytols, *Ann.*, 464: 69–90 (1928); Die Synthese des Phytols, *Ann.*, 475: 183–204 (1929).

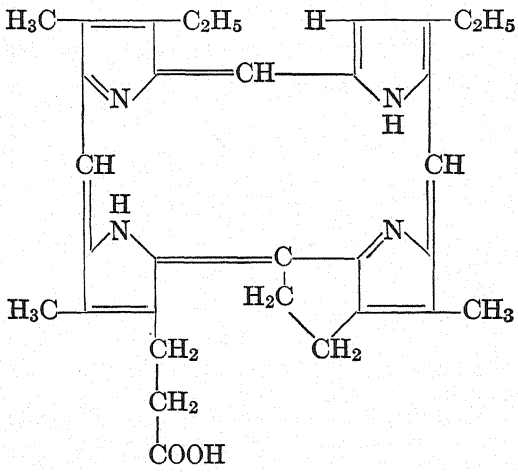
[4] Channon, H. J., and Collinson, G. A., The Absorption of the Higher Alcohols, *Biochem. J.*, 22: 391–401 (1928).

The heterocycle which may be regarded as the nucleus of both chlorophyll-*a* and chlorophyll-*b* is *phorbin*:



Phorbin

The porphin corresponding to phorbin, but lacking carbon-9 and carbon-10 and with a double bond between carbon-5 and carbon-6, and a hydrogen on the γ-carbon, has been synthesized.[5] 3-Desmethyldesoxyphylloerythrin is a somewhat more complex porphin derivative which possesses intact a number of groupings characteristic of chlorophyll. It has been synthesized by Hans Fischer,[6] and the synthetic product has been found to be identical in all its properties with the decomposition product obtained from chlorophyll.

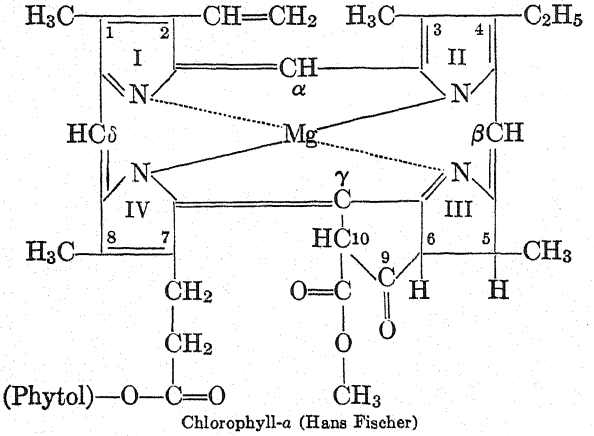


3-Desmethyldesoxyphylloerythrin

[5] Rothemund, P., A New Porphyrin Synthesis. The Synthesis of Porphin, *J. Am. Chem. Soc.*, 58: 625–627 (1936); *cf.* also Fischer, H., and Gleim, W., Synthese des Porphins, XXXV, *Ann.* 521: 157–160 (1935).

[6] Fischer, H., and Rose, W., Synthesen der β-freien Desoxophyllerythrine-1,2,3,4 und eines isomeren Desoxophyllerythrins, *Ann.*, 519: 1–42 (1935).

On the basis of these structures and many other related decomposition products of chlorophyll, Fischer [7] presents the following structural formula for chlorophyll-*a*:



Chlorophyll-*a* (Hans Fischer)

He [8] notes that chlorophyll-*b* differs from chlorophyll-*a* only in having the $—CH_3$ group on carbon-3 replaced by a —CHO group.

Stoll and Wiedemann [9] claim to have converted chlorophyll-*b* into chlorophyll-*a*. The sequence of reactions which they used were:

$$\text{Chlorophyll-}b \xrightarrow[-\text{phytol}]{-\text{Mg}} \text{phaeophorbid-}b \xrightarrow{-H_2O} \text{anhydrophaeophorbid-}b \xrightarrow{+H_2}$$

$$\text{phaeophorbid-}a \xrightarrow[\text{Grignard}]{\text{Mg}} \text{chlorophyllid-}a \xrightarrow[(\text{chlorophyllase})]{\text{phytol}} \text{chlorophyll-}a.$$

Certain of the decomposition products of chlorophyll are extremely resistant to decay. Treibs [10] isolated from oil schists (Alpine Triassic) a compound which he identified as desoxyphylloerythrin which has the same structure as 3-desmethyldesoxyphylloerythrin, except that a $—CH_3$ group replaces the hydrogen on carbon-3. The same product was also identified in bituminous earths, mineral oils, and waxes, and in asphalts, indicating that these had been derived from plant remains.

[7] Fischer, H., and Breitner, S., Vergleichende Oxydation des Chlorophyllids und einiger Abkömmlinge, *Ann.*, 522: 151–167 (1936).

[8] Fischer, H., and Bauer, K., Purpurine, Rhodine und Rhodin-porphyrine aus Chlorophyll b. Neue Analogien zwischen Chlorophyll a und b, *Ann.*, 523: 235–284 (1936).

[9] Stoll, A., and Wiedemann, E., Überführung von Chlorophyll b in Chlorophyll a, *Naturwissenschaften*, 20: 889 (1932).

[10] Treibs, A., Über das Vorkommen von Chlorophyllderivaten in einem Ölschiefer aus der oberen Trias, *Ann.*, 509: 103–114 (1934); and Chlorophyll- und Häminderivate in bituminösen Gesteinen, Erdölen, Erdwachsen und Asphalten, *Ann.*, 510: 42–62 (1934).

Many hypotheses have been proposed to account for the photosynthesis of carbohydrates through the agency of light and chlorophyll. One of the more recent of these is that of Stoll,[11] who postulates a series of five consecutive reactions:

1. The combination of carbonic acid with chlorophyll to form a chlorophyll-carbonic acid complex.
2. The transfer of the carbonic acid to hydrogen acceptors. *This is photochemical reaction I* and is a mechanism similar to a peroxidative rearrangement.
3. The dehydrogenation of the chlorophyll with a stepwise reduction of the carbonic acid.
4. The cleavage of the water molecule attached to the chlorophyll, $H_2O \rightarrow H + OH$, $2OH \rightarrow H_2O_2$, whereby the chlorophyll again becomes saturated with hydrogen. *This is photochemical reaction II.*
5. The removal by leaf catalase of the hydrogen peroxide formed in reaction 4. This is a reaction dependent upon temperature and results in the liberation of oxygen.

It will be noted that in Stoll's mechanism two separate photochemical reactions are postulated. A photochemical reaction is characterized by having a negligible temperature coefficient, and photosynthesis has been shown to be relatively unaffected by temperature in those temperature ranges which do not interfere with other vital mechanisms of the plant. Certain workers have reported some effect of temperature on the photosynthetic process. Stoll's steps 1, 3, and 5 should be influenced by temperature, and whenever they are the determining factors, temperature should affect the photosynthetic process, if Stoll's mechanism is correct.

Adams,[12] from a purely theoretical standpoint, earlier postulated a sequence of four reactions, two of which were photochemical and two of which required water and carbon dioxide but not light. Rather interestingly he also postulated the formation of hydrogen peroxide in order to account for the energy absorbed and utilized in the photosynthetic mechanism. He postulated that: (1) chlorophyll unites with water, using two quanta [13] of energy of λ 666 mμ to produce a reduced chlorophyll and hydrogen peroxide; (2) the reduced chlorophyll then combines with carbon dioxide to form a chlorophyll-carbon

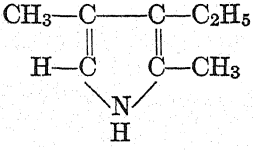
[11] Stoll, A., Über den chemischen Verlauf der Photosynthese, *Naturwissenschaften*, 20: 955–958 (1932).

[12] Adams, E. Q., The Efficiency of Photosynthesis by *Chlorella*, *J. Am. Chem. Soc.*, 48: 292–294 (1926).

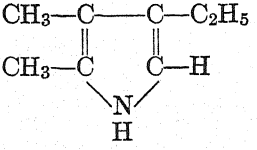
[13] Note that a quantum is an energy-time concept. It is the unit of action and is equal to 6.55×10^{-27} erg second. It is a *force* acting through *time*, and accordingly the *time* of the light vibration enters into the quantity; cf. Eddington, A. S., The Nature of the Physical World, The Macmillan Company, New York (1929), Cambridge University Press, England (1929).

dioxide complex; (3) the complex formed in reaction (2) is further reduced by combining with water under the energy of two quanta of λ 640 mμ to produce a chlorophyll-carbon monoxide complex and hydrogen peroxide; and (4) the chlorophyll-carbon monoxide complex in the presence of water breaks down to regenerate chlorophyll and form active formaldehyde.

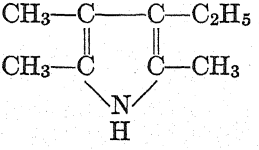
The reduction of chlorophyll yields certain nitrogen compounds, substituted pyrrole rings. The following have been identified:



(1) Kryptopyrrole



(2) Isohemopyrrole



(3) Phyllopyrrole

All three are derived from both hemin, the red pigment of hemoglobin, and from chlorophyll.

If one compares the formula of chlorophyll-*a* with the formula of hematin (Chap. XVII), it will be observed that there is a very striking similarity in the structure of these two pigments, the one the vital pigment of the autotrophic plants, the other the vital pigment of most representatives of the animal kingdom. The same porphyrin nucleus occurs in both pigments, and the groups which are attached to the pyrrole rings show a great similarity. Thus, in the case of hematin, carbon-4 carries an allyl residue, and this is an ethyl residue in chlorophyll. Carbon-6 and carbon-7 in both compounds carry a three-carbon chain, *n*-propionic acid in hematin which apparently has been somewhat modified in chlorophyll-*a*, so that in reality the structures contain almost identical groupings. It is highly improbable that the close similarity in the structure of these two vital pigments is one of chance, but it appears more probable that, in the processes involved in organic evolution, the essential nucleus of the earlier vital pigment, chlorophyll, became modified so as to assume new functions in the developing animal kingdom, the *magnesium* in the chlorophyll being replaced with *iron* in order to care for the new function as an oxygen carrier, the branched-chain aliphatic alcohol, phytol, being similarly replaced by a protein residue (a histone, globin), possibly because the animal body cannot synthesize such compounds as phytol but can reconstruct a protein molecule from the amino acids which are secured from the food.

Magnesium is an integral constituent of the chlorophyll molecule. Prior to the demonstration of magnesium in chlorophyll, it was well known that magnesium was an essential plant food, but the presence of magnesium in chlorophyll was the first demonstrated function of

the element. Apparently it should be as essential as is either phosphorus or potash. In chlorosis, magnesium may enter as well as other factors. Magnesium-deficient soils have been reported.[14]

Schertz [15] notes that in the United States alone more than 6,000,000 tons of chlorophyll are produced each year by the corn and small grain crops. He also notes that it possesses some commercial importance and that, in 1924, 3,213 pounds of chlorophyll, valued at $5,799, were imported into the United States to be used in medicines and as coloring for candles, waxes, resins, oils, soaps, foods, etc.

THE CAROTENOIDS [16]

The carotenoids are those light-yellow to deep-red nitrogen-free pigments of the plant and animal kingdoms which can be extracted from the tissues in which they occur by means of fat solvents. Most are derivatives of a highly unsaturated hydrocarbon having the general formula $C_{40}H_{56}$. Nearly all members of the group possess a carbocyclic ring or a semicarbocyclic configuration attached to each end of a highly unsaturated poly-ene hydrocarbon chain —$C_{22}H_{26}$— as shown in the formula for β-β'-carotene, connecting the carbocyclic groups.

In 1930, practically nothing was known of the structural chemistry of the carotenoids. In the last ten years, due primarily to the work of Karrer, Richard Kuhn, Zechmeister, and a number of other workers, the structures of a very considerable number of the carotenoids have been definitely ascertained. A few years ago it was believed that there were only a few examples of the carotenoid group. Today there is every indication that there are many carotenoids, nearly all of which possess some modification of the basic carotenoid structure, and

[14] Garner, W. W., McMurtrey, J. E., and Moss, E. G., Sand Drown, a Chlorosis of Tobacco and Other Plants Resulting from Magnesium Deficiency, *Science*, 56: 341–342 (1922); Garner, W. W., McMurtrey, J. E., Bacon, C. W., and Moss, E. G., Sand Drown, a Chlorosis of Tobacco Due to Magnesium Deficiency and the Relation of Sulfates and Chlorides of Potassium to the Disease, *J. Agr. Research*, 23: 27–40 (1923); *cf.* also Jones, J. P., Deficiency of Magnesium the Cause of a Chlorosis in Corn, *J. Agr. Research*, 39: 873–892 (1929).

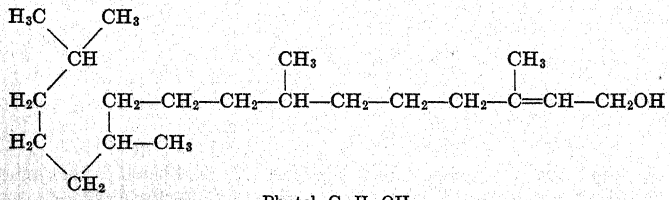
[15] Schertz, F. M., Commercial Applications of Chlorophyll Derivatives, *Ind. Eng. Chem.*, 19: 1152–1153 (1927).

[16] For essential literature see: Palmer, L. S., Carotinoids and Related Pigments, American Chemical Society Monograph, No. 9, Chemical Catalog Company, New York (1922); Zechmeister, L., Carotinoide, Julius Springer, Berlin (1934); Willstaedt, H., Carotinoide Bakterien- und Pilzfarbstoffe, Ferdinand Enke, Stuttgart (1934); Karrer, P., and Wehrli, H., 25-Jahre Vitamin-A-Forschung, *Nova Acta Leopoldina*, Neue Folge, 1: 175–339 (1933); Karrer, P., Über einige natürlich vorkommende, biochemisch bemerkenswerte Pigmente, *Helv. Chim. Acta*, 19 (Fasciculus Extraordinarius): E33–E48 (1936); and Mayer, Fritz, Chemie der organischen Farbstoffe, Bd. II, Natürliche organische Farbstoffe, Dritte umgearbeitete Auflage, Julius Springer, Berlin (1935).

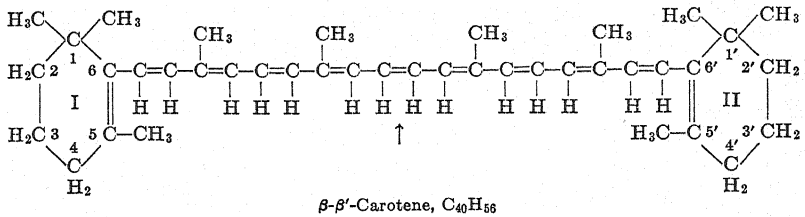
these modifications reside almost wholly in the configuration of the carbocyclic or semicarbocyclic groupings situated at the two ends of the molecule. In the *Annual Review of Biochemistry* for 1937 there are listed fourteen carotenoids, the isolation of which has been reported but the structures of which are still somewhat uncertain. These are in addition to the compounds noted in Table LXXVIII, and a great many other carotenoids have been indicated in the literature. It appears probable, therefore, that there may be as many as one hundred different compounds belonging to the carotenoid group.

The first carotenoids to be extensively studied and somewhat characterized were designated "carotin" and "xanthophyll," which were prepared in relatively pure form by Willstätter during his studies of chlorophyll. Following these studies a number of workers studied some phases of the carotenoid problems, but little progress was made on the structural chemistry until Karrer undertook his investigations. Karrer showed that one of the components of "carotin," *i.e.*, β-carotene, was the provitamin of vitamin A (*vide infra*), and that vitamin A apparently arises in the animal body by the cleavage of the β-β'-carotene molecule at the double bond in the middle of the molecule, the terminal carbon being converted into a primary alcohol. Thus, β-β'-carotene, on cleavage and simultaneous oxidation and reduction, gives rise to two molecules of vitamin A, $C_{40}H_{56} + 2H_2O = 2C_{20}H_{30}O$. *Only those carotenoids which contain the β-carotene grouping can act as precursors for vitamin A.* Four of the natural pigments are recognized as containing this grouping, *i.e.*, α-, β-, and γ-carotene, and cryptoxanthin. Those carotenoids which contain substitutions in the carbocyclic ring of β-carotene are inactive so far as transformation into vitamin A is concerned. Each molecule of β-carotene yields two molecules of vitamin A; each molecule of α-carotene, γ-carotene, and cryptoxanthin yields only one molecule of vitamin A, since only one unsubstituted β-carotene residue is present in these molecules.

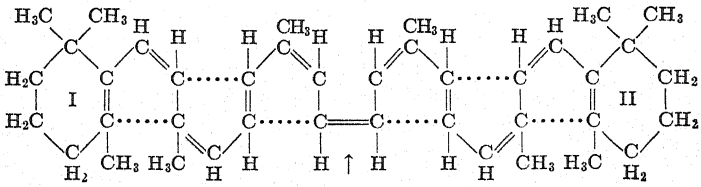
Little is known in regard to the origin of the carotenoids in nature. It seems possible, however, that they are genetically related to the aliphatic alcohol phytol, which is esterified on the chlorophyll molecule. When the structural formula of phytol is written with a carbon arrangement similar to the carbon arrangements in the carotenoid molecules, there is a striking resemblance to the carotenoid structure.



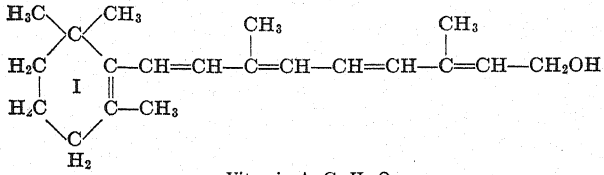
Phytol, $C_{20}H_{39}OH$



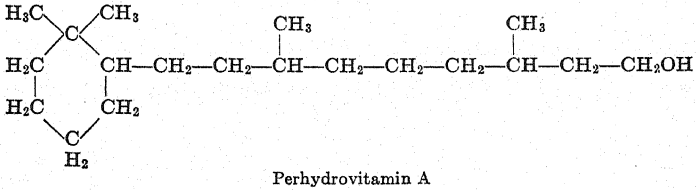
β-β′-Carotene, $C_{40}H_{56}$



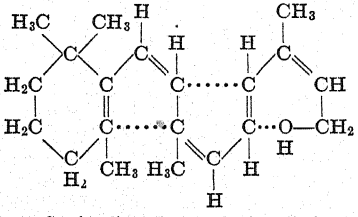
Palmer's semicyclic structure of β-β′-Carotene



Vitamin A, $C_{20}H_{30}O$



Perhydrovitamin A



Semicyclic structure of vitamin A

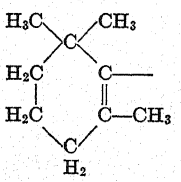
The structure of β-β′-carotene is written in two ways. The straight-chain formula is the conventional form. Palmer [17] suggests the semicyclic structure as more probable, and the author agrees with

[17] Palmer, L. S., personal communication.

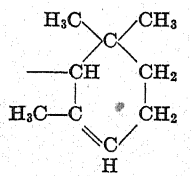
this suggestion. In all probability covalence forces as indicated tend to hold the molecule in the semicyclic form. The arrow in the formulas for β-β'-carotene indicates the double bond in the middle of the molecule where the break occurs to form two molecules of vitamin A.

NOMENCLATURE.—In the chemical literature nearly all the carotenoids have been given a name, in many instances derived from the biological material from which the compound was isolated. Palmer [18] has proposed a series of chemical names to indicate the relationships of one carotenoid to another. He has retained the name β-carotene as indicating a *hemi*-carotenoid possessing an *optically inactive* carbocyclic residue which has the structural relationships of β-ionone. α-Carotene he uses to designate a *hemi*-carotenoid possessing the carbocyclic structure characteristic of α-ionone and containing an *optically active* asymmetric carbon atom. For other carbocyclic structures β- is used to designate the *optically inactive* form and α- the *optically active* configuration. The lycopene structure is different from that of α- and β-ionone in that the semicarbocyclic ring is not closed, and therefore Palmer retained "lycopene" to designate the "lycopene residue." Similarly the semicarbocyclic residue of fucoxanthin is not closed, and the author, in consultation with Palmer, proposes α-fucoxanthin to designate the fucoxanthin residue which contains an asymmetric carbon atom.

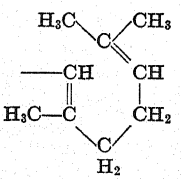
Using this nomenclature it is not necessary to present detailed structural formulas of most members of the carotenoid series, since the $—C_{22}H_{26}—$ unsaturated hydrocarbon chain which links the carbocyclic or semicarbocyclic residues together is common to all the group. Therefore, referring to the structural formula of β-β'-carotene, this could be designated as (I)$—C_{22}H_{26}—$(II), where, in the case of β-β'-carotene, I and II represent the β-ionone residue. The other residues which have been identified as occurring in the various carotenoids are indicated below.



β-Ionone residue (at I in α-carotene, in γ-carotene, in vitamin A, at I and II in β-carotene, at II in cryptoxanthin)

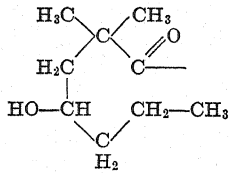


α-Ionone residue (at II in α-carotene and δ-carotene)

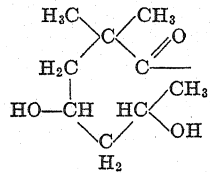


β-Lycopene residue (at I and II in lycopene, at I in δ-carotene, and at II in γ-carotene and in rubixanthin)

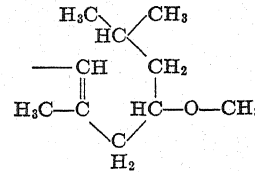
[18] Palmer, L. S., The Biological and Chemical Nomenclature for the Carotenoids, *Science*, 79: 488–490 (1934).



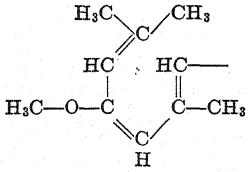
Desoxy-α-fucoxanthin residue (at I and II in capsorubin and at II in capsanthin)



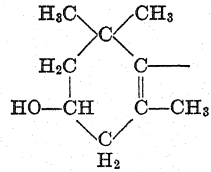
α-Fucoxanthin residue (at I and II in fucoxanthin)



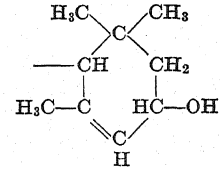
3-Methoxy-1-2-dihydro-β-lycopene residue (at II in rhodoviolascin)



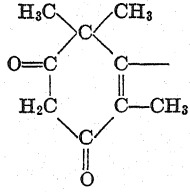
3-Methoxy-Δ34-dehydro-β-lycopene residue (at I in rhodoviolascin)



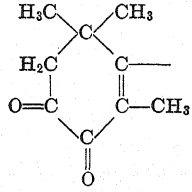
3-Hydroxy-β-carotene residue (at I in xanthophyll, capsanthin, rubixanthin, cryptoxanthin, and helenien, and at I and II in physalien and zeaxanthin)



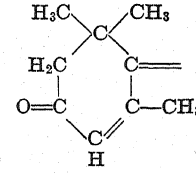
3-Hydroxy-α-carotene (at II in xanthophyll and helenien)



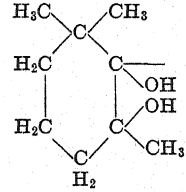
2-4-Diketo-β-carotene residue (at I and II in euglenarhodon)



3-4-Diketo-β-carotene residue (at I and II in astacene)



3-Keto-Δ45-β-carotene residue (at I and II in rhodoxanthin)



1-2-Dihydroxydihydro-α-carotene residue (in azafrin)

Using this nomenclature, Table LXXVIII lists most of the naturally occurring carotenoids, the structures of which are known, together with their common names, the names indicating structural relationships, and some of the sources from which they have been obtained.

THE CAROTENOIDS OF THE TISSUES OF HIGHER ANIMALS.—Palmer [19] and Eckles were largely responsible for demonstrating that the so-

[19] Palmer, L. S., Carotinoids and Related Pigments.

called "lypochromes" of animal tissues are in reality carotenoids which originated in the food and which have been deposited in the fat and adipose tissues of the animal. Thus, they showed that the yellow pigment of butterfat is almost wholly carotene and that both carotene and "xanthophyll" occur in the fat of human milk. Carotene admixed with some "xanthophylls" was found by them to be the pigment in the adipose tissue of cattle, whereas they found the pigment of the yolk of hen's eggs to be almost exclusively "xanthophyll."

TABLE LXXVIII

CERTAIN OF THE NATURALLY OCCURRING CAROTENOIDS

Common Name	Name Indicating Structural Relationships	Occurrence
α-Carotene.....	β-α′-Carotene	Carrots, palm oil, chestnuts, mountain ash berries
β-Carotene.....	β-β′-Carotene	Carrots, paprika, green leaves, grass, etc.
γ-Carotene.....	β-Lyco-β′-carotene	Apricots, fruits of *Convallaria majalis* and *Gonocaryum pyriforme*
δ-Carotene.....	β-Lyco-α′-carotene	Fruits of *Gonocaryum pyriforme*
Lycopene.....	β-β′-Lycopene	Fruit of tomato (*Lycopersicum esculentum*), watermelon (*Cucumis citrullus*), etc.
Rhodoxanthin....	3-3′-Diketo-4-4′-dehydro-β-β′-carotene	Fruit of the yew, *Taxus baccata*, leaves of *Potamogeton*, etc.
Xanthophyll Lutein }	3-3′-Dihydroxy-β-α′-carotene	Green leaves, corpus luteum, many flowers
Zeaxanthin.....	3-3′-Dihydroxy-β-β′-carotene	Yellow *Zea mays*
Fucoxanthin.....	α-α′-Fucoxanthin	Brown algae, *Fucus* sp.
Cryptoxanthin....	3-Hydroxy-β-carotene-β′-carotene	Fruits of *Carica papaya*, yellow *Zea mays*, *Capsicum* sp., etc.
Capsanthin.....	3-Hydroxy-β-carotene-5′-desoxy-α′-fucoxanthin	Ripe fruits of *Capsicum annuum*
Helenien.....	β-α′-carotene-3-3′-dioldipalmitate	Flowers of *Helenium* sp., *Tagetes* sp., etc.
Physalien.....	β-β′-carotene-3-3′-dioldipalmitate	Fruits of *Physalis alkekengi* and *P. franchetti*, asparagus, etc.
Astacene.....	3-3′-4-4′-Tetraketo-β-β′-carotene	The carapace of the *Crustacea*, starfish, crabs, etc.
Capsorubin.....	5-5′-Desoxy-α-α′-fucoxanthin	Ripe fruits of *Capsicum annuum*
Euglenarhodon...	2-2′-4-4′-Tetraketo-β-β′-carotene	*Euglenia* sp.
Rubixanthin.....	3-Hydroxy-β-carotene-β′-lycopene	Rose hips, *Rosa* sp.
Rhodoviolascin...	3-3′-Dimethoxy-Δ34-dehydro-1′-2′-dihydro-β-β′-lycopene	Purple bacteria, *Rhodovibrio* and *Thiocystis*

Palmer, and Palmer and Eckles have demonstrated that the carotene content of the cow's tissues, as well as that secreted in the milk fat, is determined by the carotene content of the ration. When foods rich in "xanthophyll," such as yellow maize, were fed, no appreciable pigmentation of the animal tissues or of the milk secretion took place, indicating that the xanthophyll and zeaxanthin in the food were not transported unchanged through the blood stream to the various tissues, in contrast to the behavior of carotene. Similar experiments by Palmer, and Palmer and Kempster showed that "xanthophyll-rich" rations

increased the pigmentation of egg yolk, whereas carotenoid-poor rations or rations in which carotene was the only carotenoid present caused the production of carotenoid-free egg yolks. The "xanthophyll-rich" rations produced a rapid coloration in all parts of the body of the white leghorn fowl, whereas carotene-containing foods had practically no effect upon the coloration of the bird's tissues. Palmer and Kempster were able to rear chickens to maturity on diets which were devoid of the carotenoids that are normally present in the adipose tissue of fowls. The eggs of such chickens possessed carotenoid-free yolks, but this fact did not impair their fertility, nor were the young chickens hatched from such eggs inferior to those hatched from normally pigmented eggs. The success of the experiments of Palmer and Kempster is explained by the fact that they included an adequate amount of (colorless) vitamin A (pork liver) in their rations.

The yellow pigment of the corpus luteum, as well as the corpus rubrum, is almost wholly β-β'-carotene. The human placenta contains both carotene and xanthophyll. The adrenal glands contain carotene both in the medulla and in the cortex, and we have already indicated that a carotenoid protein is the visual purple of the retina.

Astacene, the carotenoid of the Crustacea, crystallizes from aqueous pyridine in violet needles which possess a metallic luster. It forms a blood-red solution in pyridine which is orange-red when dilute. It apparently exists in the carapace of the Crustacea in the form of a chromoprotein or perhaps adsorbed upon protein. When the protein is denatured with heat or with alcohol, the red astacene is liberated from the brownish-green chromoprotein. This accounts for the change in color when lobsters are boiled.

The Identification and Separation of Carotenoids.—Tswett, in 1906, introduced a special adsorption technic, or rather adsorption analysis method, which he called the chromatographic analysis, and this technic has been used almost exclusively in the separation of the various carotenoids. It consists essentially in the adsorption of the carotenoids upon calcium carbonate, calcium hydroxide, gypsum, magnesium oxide, aluminum oxide, or other finely divided powders. The powder is packed uniformly in a glass tube, and the carotenoid-containing solution is allowed to percolate through the mass of packed powder. Those carotenoids or other substances which are most strongly adsorbed by the solid powder form a colored band or zone near the top of the powder column. Under proper conditions of concentration of solution, etc., this upper band or zone is followed by a band of the powder free from adsorbed constituents. If only one compound is present in the solution being passed through the powder, only a single uniform adsorption band will form. If the solution being passed through contains several components, the chromatograph will consist of a series of bands somewhat similar to that shown in Fig. 157.

Following the adsorption of the desired constituents, the column of powder is removed from the tube and separated into the various fractions by cutting the column between the colored bands. The pigment is then elutriated from the powder and usually readsorbed until it is definitely certain that the elutriated fraction consists of a single component. This fraction is then elutriated and studied by appropriate technics. In many of the carotenoids it is very essential that the adsorption and elutriation, as well as the subsequent study of the carotenoid, be carried out in the absence of oxygen, since many of the carotenoids are very subject to oxidation. Zechmeister and Cholnoky [20] have outlined in detail the chromatographic technic. A given compound may be adsorbed from one solvent and elutriated by another solvent. A mixture of compounds shows, as would be anticipated, very different chromatographs depending upon both the solvent used and the solid material upon which the pigments are adsorbed.

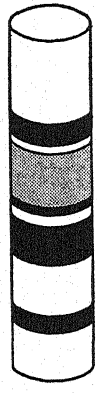


FIG. 157.—A diagrammatic representation of the appearance of the colored adsorption bands in a Tswett "chromatographic analysis" tube.

THE ROLE OF CAROTENOIDS IN PLANTS.—There is no general agreement as to the function of the carotenoids in plants. The fact that they invariably occur associated with chlorophyll in the chloroplasts would permit the assumption that they play some role in the photosynthetic process. They show characteristic absorption bands which would permit of their absorbing light of certain wave lengths and converting this energy to chemical uses. This may be their more important function; or it may be that they act as an oxidation ⇆ reduction system and are concerned with oxygen transference or oxygen assimilation. Willstätter and Stoll suggest that one possible function of the carotenoids may be to control the equilibrium between chlorophyll-*a* and chlorophyll-*b*. Other suggestions of physiological function have been made, but as yet there is no proof that they play a major role in any single physiological mechanism.

THE ABSORPTION SPECTRA OF THE CAROTENOIDS.—Each one of the carotenoids possesses a specific and distinct absorption spectrum which is measurable in extremely great dilutions. Thus, wheat flour probably does not contain more than 2.5 parts of carotenoid per million parts of flour. The absorption bands of the carotenoid solutions are, however, so distinct and so characteristic that a quantitative determina-

[20] Zechmeister, L., and Cholnoky, L., von, Die chromatographische Adsorptionsmethode, Julius Springer, Wien (1937).

tion of the carotenoid content of wheat flour can be made on the extract of as little as 1 gram of flour. The monograph by Zechmeister may be consulted for the position of the specific absorption bands.

Carotenoid studies afford a very striking illustration of how a pure science study, which apparently had no practical importance, may, in an emergency, be found to be of practical use. Prior to the World War the absorption spectra of the carotenoids and of chlorophyll had been exhaustively studied and mapped. During the World War it became necessary for those in charge of the American troops on the western front to devise some means for the detection of enemy troop movements which were being carried out under camouflages so designed as to simulate green foliage. Natural green foliage reflects both red and green light, owing to the fact that carotenoids and chlorophyll are both present in the chloroplasts. Inasmuch as the quantity of chlorophyll greatly exceeds the quantity of the carotenoids, the foliage appears to the eye as a more or less pure green, the reds and yellows of the carotenoids being masked. Accordingly it was possible to construct covered roads for the movement of troops, the roads being camouflaged to represent an integral part of the landscape by having the covering painted with green paint. To the eye of an observer in an aeroplane, such camouflaged roads would appear to merge with the green of the surrounding fields.

The Eastman Kodak Company devised a light filter capable of screening out the green rays characteristic of chlorophyll but permitting the wide band in the red at about 700 mμ and a narrow band in the green at about 500 mμ, bands characteristic of the carotenoids, to pass through the filter. Accordingly, when a landscape is viewed through such a filter, the natural vegetation does not appear green but instead as a more or less bright red or orange-red,[21] giving an appearance as if the landscape were on fire. A strip of green paint in such a field still appears green when viewed through such a ray filter, inasmuch as the paint pigment does not possess the characteristic optical properties of chlorophyll. Accordingly, when a landscape was observed, from an aeroplane, to have the appearance of a prairie fire and a thin green line appeared across such a landscape, it was easy to determine exactly where to place the aeroplane bombs.

No one could have predicted that research dealing with the absorption spectra of chlorophyll and the carotenoids would ever be turned to practical use, and there is no reason for believing that many of the pure science studies which have been or are being carried out in other fields may not have similar or even greater practical importance.

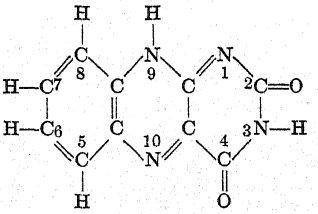
[21] The summer foliage, viewed through this filter, has the brilliant coloration characteristic of the autumn days.

CHAPTER XXXI

THE FLAVINS

The flavins are a group of yellow pigments possessing marked fluorescence which are widely distributed in both plant and animal tissues and which may be a constituent of all living cells. They have recently assumed extraordinary importance in that vitamin G (vitamin B_2) (*vide infra*) has been shown to be a flavin, and the yellow oxidation-reduction enzyme of Warburg and Christian is a flavin-phosphoric acid-protein complex.

Flavins are derivatives of the heterocyclic *iso*alloxazine nucleus:

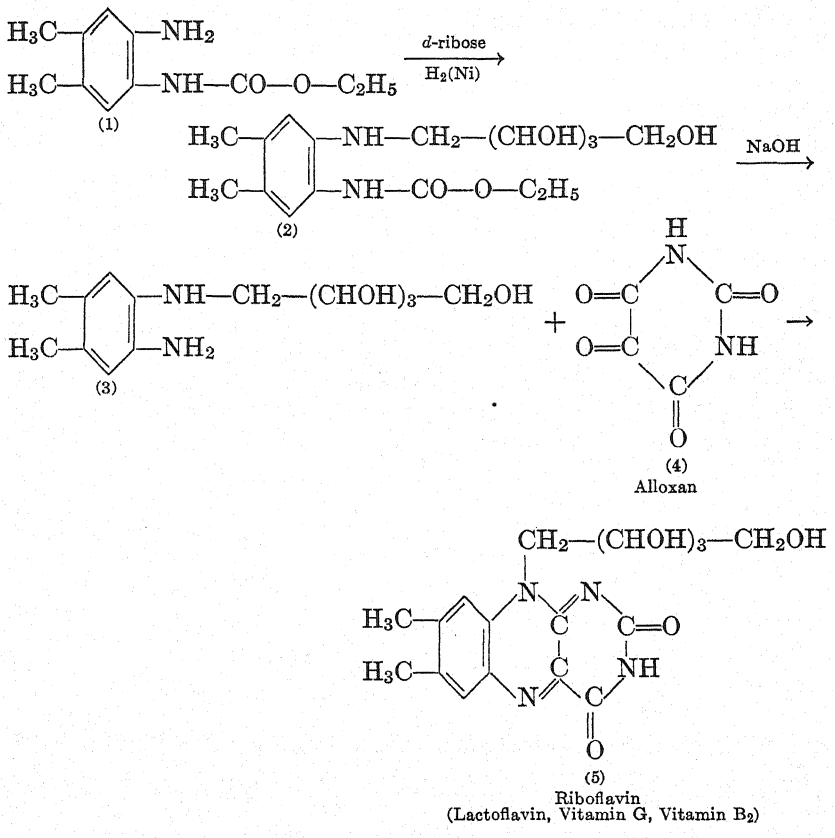


*Iso*alloxazine

Riboflavin (lactoflavin or ovoflavin or vitamin G) is 6-7-dimethyl-*iso*alloxazine-9-*d*-riboside. It is the water-soluble, yellow (with a greenish fluorescence) pigment which occurs in milk whey, and in both egg yolk and egg white. Following its identification in these substances it has been shown to occur in green leaves and in a great variety of plant and animal tissues. Its structure was definitely ascertained by its almost simultaneous synthesis by Karrer [1] and by Kuhn.[2] In Karrer's synthesis 2-amino-4-5-dimethylcarbethoxy analide was (1) treated with *d*-ribose under reducing conditions to form the corresponding amino riboside (2). The carbethoxy group on the other amino group was then saponified with alkali and the resulting riboside (3) was condensed with alloxan (4) to form riboflavin (5).

[1] Karrer, P., Schöpp, K., and Benz, F., Synthesen von Flavinen IV, *Helv. Chim. Acta*, 18: 426–429 (1935).

[2] Kuhn, R., Reinemund, K., Kaltschmitt, H., Ströbele, R., and Trischmann, H., Synthetisches 6-7-Dimethyl-9-*d*-ribo Flavin, *Naturwissenschaften*, 23: 260 (1935).



It was identical in all respects with the natural product. Riboflavin, when acted on by light under alkaline conditions, gives rise to lumiflavin (6-7-9-trimethyl*iso*alloxazine). Under neutral conditions, in methanol, lumichrom (6-7-dimethylalloxazine) results.

Karrer has synthesized a very considerable number of flavins more or less analogous in structure to riboflavin. Analogs having the following groups at (9) have been synthesized: *d*- and *l*-arabinose, *d*- and *l*-ribose, *d*-xylose, *d*-lyxose, *l*-rhamnose, *d*-desoxyribose, as well as other non-sugar residues, such as certain of the glycols. The following compounds all showed pronounced vitamin-G activity:

6-7-Dimethyl-9-*d*-ribityl*iso*alloxazine
6-7-Dimethyl-9-*d*-arabityl*iso*alloxazine
6-Ethyl-7-methyl-9-*d*-ribityl*iso*alloxazine
7-Monomethyl-9-*d*-ribityl*iso*alloxazine
6-Monomethyl-9-*d*-ribityl*iso*alloxazine

In nature riboflavin appears to be esterified with phosphoric acid, and this riboflavin phosphate is attached to a protein carrier.

THE YELLOW RESPIRATION ENZYME.—Respiration enzymes may be divided into two general groups: (*a*) those whose activity is inhibited by HCN and carbon monoxide, and (*b*) those which are not inhibited by HCN and carbon monoxide. Those enzymes of group (*a*), such as cytochrome, are heme compounds. Those of group (*b*) include compounds which do not contain a metallic radical, and chief in this group is the yellow respiration enzyme of Warburg and Christian.[3] The yellow respiration enzyme is an oxidation $\rightleftharpoons$ reduction catalyst. It is water-soluble, in the oxidized form is yellow with a green fluorescence, and in the reduced form is practically colorless. Theorell[4] has shown that the yellow oxidation enzyme is a riboflavin-phosphoric acid-protein complex, and that the riboflavin-phosphoric acid ester can be removed from the protein and when so removed neither the protein nor the riboflavin-phosphoric acid ester separately show any enzymatic activity. When, however, the protein is added to the riboflavin-phosphoric acid ester, the original enzyme activity is regained. Kuhn[5] synthesized the yellow respiration enzyme. Kuhn's synthetic riboflavin was esterified with phosphoric acid, and this riboflavin-phosphoric acid ester plus protein had the same enzyme activity *in vitro* as the natural enzyme. The combination of the pigment with the protein increased its oxidation-reduction potential at *p*H 7 and 0° from -0.181 to -0.06 volt.

Warburg and Christian's oxidation enzyme has been isolated and studied from such diverse materials as liver, egg white, and egg yolk, kidney, malt, milk whey, grass, and the retina of the eye. In all instances riboflavin was found to be the pigmented residue.

Warburg's enzyme requires a coenzyme which acts as a hydrogen donator. This hydrogen donator in biological tissues is apparently a compound containing adenine, nicotinic-acid-amide, a sugar, and phosphoric acid. Karrer[6] notes that the methyl iodide of nicotinic acid may serve as a model for the reactions involved.[7]

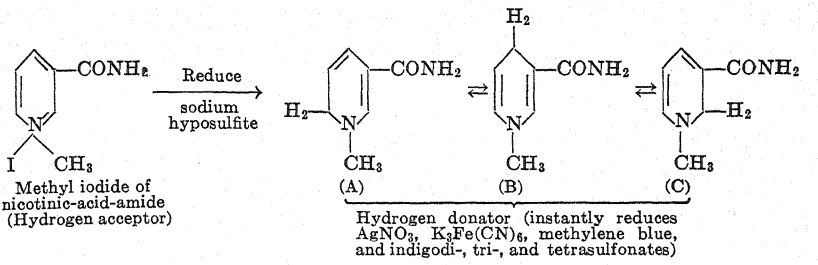
[3] Warburg, O., and Christian, W., Über ein neues Oxydationsferment und sein Absorptionsspektrum, *Biochem. Z.*, 254: 438–458 (1932); Über das gelbe Oxydationsferment, *ibid.*, 257: 492 (1933).

[4] Theorell, H., Das gelbe Oxydationsferment, *Biochem. Z.*, 278: 263–290 (1935).

[5] Kuhn, R., and Rudy, H., Katalytische Wirkung der Lactoflavin-5'-phosphorsäure; Synthese des gelben Ferments, *Ber.*, 69: 1974–1977 (1936).

[6] Karrer, P., Über einige natürlich vorkommende, biochemisch bemerkenswerte Pigmente, *Helv. Chim. Acta*, 19 (Fasciculus Extraordinarius): E33–E48 (1936).

[7] Warburg, O., and Christian, W., Pyridin, der wasserstoffübertragende Bestandteil von Gärungsfermenten, *Helv. Chim. Acta*, 19 (Fasciculus Extraordinarius): E79–E88 (1936).



The coenzyme probably goes through a sequence of reactions similar to the above.

Nicotinic acid amide compound in coenzyme $\xrightarrow[\text{donator}]{H_2 \text{ from}}$ Reduced coenzyme analogous to (*A*), (*B*), and (*C*) above $\xrightarrow[H_2]{\text{donates}}$ Leucoflavin (reduced yellow respiration enzyme) $\xrightarrow{O_2}$ Flavin (oxidized form of yellow respiration enzyme)

In the natural coenzyme a hexosephosphate may replace the methyl iodide on the nicotinic-acid-amide, and this may explain the role of hexosephosphates as coenzymes in carbohydrate fermentation. Warburg and Christian note that when a solution of a triphosphopyridine-nucleotide was added to monopotassium hexosephosphate there was no fluorescence and no reduction of the nucleotide. However, when a "carrier protein" was first added to the triphosphopyridine-nucleotide and the monopotassium hexosephosphate was added to the mixture, there was a white fluorescence characteristic of the reduced dihydropyridine compound and the nucleotide combined with the protein was reduced.

TOXOFLAVIN.—A yellow toxic bacterial pigment was identified [8] in 1933 as being the toxic principle in certain foods commonly consumed in Java. The foods which are likely to be toxic are "Bongkrek," a product prepared from coconut presscake which has been acted upon by fungi (*Rhizopus* sp.), and "Semaji," prepared from grated coconut after the pressing of the oil. From the toxic foods a yellow pigment was isolated, very soluble in water, alcohol, and fats, somewhat soluble in benzene and chloroform, and insoluble in petroleum ether. This yellow pigment was found to be extremely toxic. In later papers [9] the authors suggest that it may be related to the flavins, and they call it toxoflavin. In solution it has an intense greenish yellow color with a green fluorescence. Analytical data indicate an empirical formula, $C_6H_6N_4O_2$. It serves as an oxidation $\rightleftharpoons$ reduction system, being col-

[8] van Veen, A. G., and Mertens, W. K., On the Isolation of a Toxic Bacterial Pigment, *Proc. Acad. Sci. Amsterdam*, 36: 666-670 (1933).

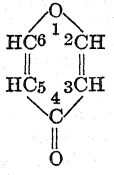
[9] van Veen, A. G., and Mertens, W. K., Die Giftstoffe der sogenannten Bongkrekvergiftungen auf Java, *Rec. trav. chim.*, 53: 257-266 (1934); Das Toxoflavin, der gelbe Giftstoff der Bongkrek, *ibid.*, 53: 398-404 (1934).

ored in the oxidized form and colorless in the reduced form. It is exceedingly toxic, the toxic dose for a rat lying between 5 and 25 μg. One to two milligrams *per os* will kill a donkey. The organism which produces this toxin is provisionally designated as the "Bongkrek" bacterium. Until further work is done on the structural chemistry of the molecule, it is impossible to say whether it is structurally related to the true flavins.

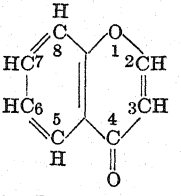
CHAPTER XXXII

THE FLAVONES, XANTHONES, ANTHOXANTHONES, AND ANTHOCYANINS

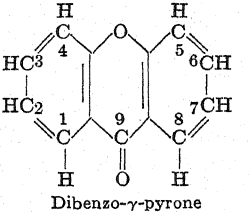
THE FLAVONES (Latin, *flavus* = yellow), XANTHONES (Greek, $\chi\alpha\nu\theta\omega\sigma$ = yellow), OR ANTHOXANTHONES.[1]—These are a class of naturally occurring plant pigments and may be regarded as phenyl derivatives of the 1.4- or γ-pyrone nucleus.



1.4- Pyrone or *γ-pyrone*



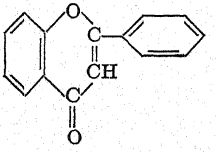
1.4- Benzopyrone or benzo-γ-pyrone or *chromone*



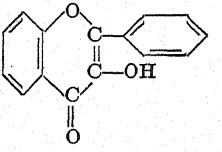
Dibenzo-γ-pyrone or *xanthone*

The keto group in these compounds does not behave as a typical carbonyl group, *e.g.*, the oxygen is not replaceable by hydroxylamine to form oximes. In addition, the ring oxygen is *basic* and adds acid as though it were *quadrivalent* oxygen, resulting in the formation of oxonium salts. The substitution of a hydroxyl group in position 3 in the chromone nucleus forms *chromonol.*

Coloring matters of the chromone and chromonol type have not as yet been found in nature, but the benzene derivatives are the *flavones* or *flavonols.* Almost all the derivatives of flavonol are yellow dyes.



Flavone (Phenylated chromone)



Flavonol (Phenylated chromonol)

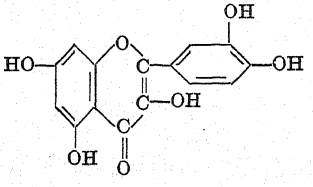
Watson [2] has brought together a considerable part of our knowledge in regard to those groups which produce color in an organic com-

[1] Possibly the term "anthoxanthones" is best, since it indicates their similarity to the anthocyanins.

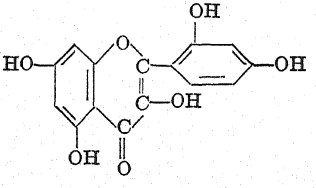
[2] Watson, E. R., Colour in Relation to Chemical Constitution, Longmans, Green and Company, London (1918).

pound and those auxiliary groups which convert a colored compound into a pigment or a dyestuff. Those groups which produce color are called *chromophore* groups. Typical chromophore groupings are the nitro group (—NO_2), the azo group (—N=N—), the quinone grouping (*ortho* or *para* O=C_6H_4=O), etc. Such groups do not necessarily produce pigments or dyestuffs. For example, azobenzene is not a dyestuff. If, however, we add one or more *auxochrome* (intensifying or modifying) groups, we obtain a dyestuff. Typical auxochrome groups are amino groups, hydroxyl groups, sulfonic acid groups, etc. In plants the organic compound containing the auxochrome groups but containing the chromophore group in a reduced or otherwise altered form, is often spoken of as a chromogen. Thus, indoxyl is a colorless *chromogen* which oxidizes to indigo blue or indigotin. The color of the indigotin can be altered by the introduction of auxochrome groups, *e.g.*, Br, NH_2, NO_2, OH, etc., into the benzene nuclei.

Quercetin, the coloring matter of quercitrin extract, is a tetrahydroxy derivative of flavonol. The *morin* of fustic (Cuba wood) differs from quercetin only in the position of the hydroxyl groups on the benzene nuclei.

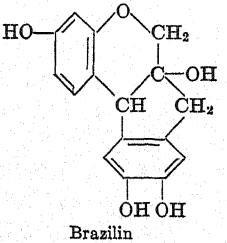


Quercetin



Morin

Brazilin, a reduced γ-pyrone derivative, is the coloring principle of hypernic. The constitution was established in 1908 by Sir W. H. Perkin, Jr., who showed it to have the formula

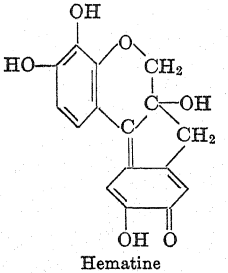


Brazilin

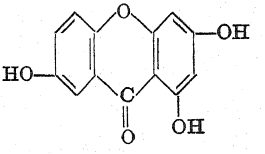
Hematoxylin, from logwood, may be regarded as a molecule of brazilin containing one additional hydroxyl group in position 8. Logwood is the source of an excellent black dye. It "weights" silk to about 200 per cent of the original weight of the silk, and the volume

of the silk is enlarged so that the fiber has greater resistance to wear.[3]

By the oxidation of hematoxylin we obtain *hematine*. In the oxidation process an additional chromophore grouping (*p*-benzoquinone) has been introduced into the molecule.



Hematine



Gentisin

The xanthone dyestuffs are not important so far as their natural occurrence is concerned. *Gentisin*, the coloring matter of the gentian root, is a trihydroxyxanthone. Only one or two other natural pigments belong to the xanthone group.

A great deal of our knowledge of the flavones is due to the work of Perkin[4] and those associated with him. The intensity of the color which this group of compounds possess depends markedly on the position of the hydroxyl groups, and as a rule the color is intensified if two hydroxyl groups are *ortho* in position to each other. This group of pigments usually occurs in plants as glycosides, one or more hydroxyl groups being combined with a sugar molecule. This has the result that the auxochrome group is rendered inactive so that *in the plant* the flavone glycosides are practically colorless. On hydrolysis of the glycoside, the color develops. Almost all white flowers turn yellow when exposed to the vapors of ammonia, owing to a salt formation of ammonium with the flavones (and possibly in part to hydrolysis of glycosides).

As a rule, the flavone type of pigments does not occur in animal tissues. Thomson,[5] however, isolated a flavone in the study of the pigments of the wings of the butterfly, *Melanargia galatea*. The flavone had the properties of quercetin, although it was not positively identified. In a later paper, Thomson[6] notes that the larva of the

[3] Chapin, E. S., Natural Dyestuffs—An Important Factor in the Dyestuff Situation, *J. Ind. Eng. Chem.*, 10: 795–798 (1918).

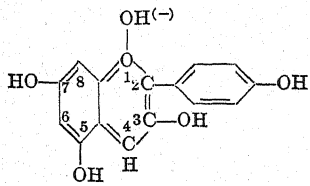
[4] Perkin, A. G., and Everest, A. E., The Natural Organic Colouring Matters, Longmans, Green and Company, London (1918).

[5] Thomson, D. L., The Pigments of Butterflies' Wings. I. *Melanargia Galatea*, *Biochem. J.*, 20: 73–75 (1926).

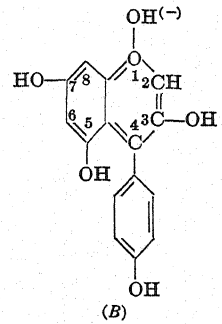
[6] Thomson, D. L., The Pigments of Butterflies' Wings. II. Occurrence of the Pigment of *Melanargia Galatea* in *Dactylis Glomerata*, *Biochem. J.*, 20: 1026–1027 (1926).

butterfly feeds upon the grass, *Dactylis glomerata*, and that this grass contains a flavone-glycoside, the flavone of which is identical with the pigment isolated from the wings of the butterfly. Apparently, therefore, this pigment of the butterfly wings has a plant and not an animal origin.

THE ANTHOCYANINS.—The red, violet, and blue pigments present in the blossoms, fruit, and leaves of many plants belong to the group of the anthocyanins. Almost invariably pigments of the above colors belong in this class. True anthocyanins are derivatives of the γ-pyran



(*A*)



(*B*)

or benzopyranol nucleus, and all, or practically all, that have been studied are derivatives (by the introduction of additional hydroxyl or methoxy groups) of the compound possessing formula (*A*), although Nierenstein [7] suggests that the formula may be as shown in (*B*). For the present we will adhere to the older formula (*A*). Formula (*A*) can be designated as 3-5-7-trihydroxy-2-*p*-hydroxyphenyl-1-4-benzopyranolanhydrohydroxide. This is the formula of *pelargonidin*. The positions on the phenyl substituted position 2 may be conveniently designated as *o*- and *o'*-, *m*- and *m'*-, and *p*-.

The chromophore group in this formula appears to be that of a substituted *ortho*-benzoquinone. The anthocyanins are further characterized by the heterocyclic oxygen functioning as *tetravalent oxygen*, *i.e.*, the anthocyanin compounds readily add acids to form *oxonium salts*. Solutions of the anthocyanins act as *indicators*, the anthocyanin molecule readily combining with acids to form compounds where *the negative radical is bound to the oxygen*. All or nearly all anthocyanins are *red* (or purple) in acid solution and possess green or blue shades in neutral or alkaline solution.

The anthocyanins are very widely distributed throughout the plant kingdom, the majority of the higher plants containing anthocyanin at some stage in their development. It is to anthocyanins that we owe

[7] Nierenstein, M., The Constitution of Catechin. IX. Some New Disintegration Products of Acacatechin, *J. Am. Chem. Soc.*, 48: 1959–1975 (1926).

the brilliant colors of our flowers, our foliage plants, and our autumn landscapes.

The function of anthocyanins in the plant is a subject which is still open to speculation. They are apparently rather easily reduced and oxidized, and it has been suggested by many, notably Palladin, that they are respiratory pigments and act as oxygen carriers, assisting in cell oxidations and reductions. This, however, still remains to be definitely proved.

The Alpine plants are particularly rich in anthocyanins and lose much of their color when grown at lower levels. One suggestion as to a possible function is that they may serve to screen out the injurious short wave lengths of light which pass through the thin atmosphere at the higher elevations. The pigment also acts as an absorber of the heat rays. Leaves colored with anthocyanins may be as much as 2° C. warmer than other leaves on the same plant which contain only the green pigment and which have the same light exposure.

Of recent years botanists and geneticists have turned to the inheritance of color in plants in order to study the laws governing heredity. Color is easy to see and to follow in the progeny through successive generations; therefore, the inheritance of color has been studied. The early workers did not appear to realize that what they were in reality studying was the *inheritance of chemical compounds and the inheritance of chemical reactions* where more than one component was involved. Miss Wheldale [8] (Mrs. Onslow) pointed this out, and later Keeble and Armstrong [9] and Keeble, Armstrong, and Jones [9] added further details, so that we now have a fairly good idea of the chemistry of color inheritance in certain flowers.

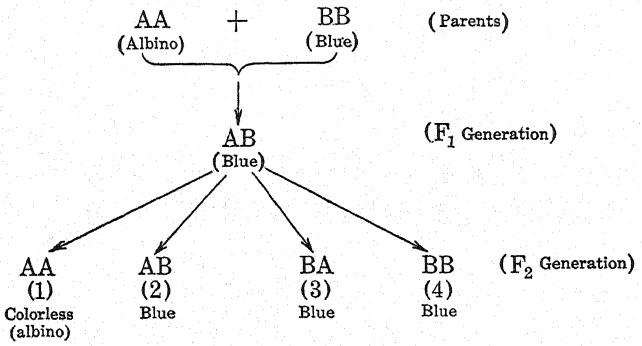
Mendel's law of inheritance has been found to apply to the inheritance of color in flowers. The only method of learning the genetic factors involved is by crosses between parents which differ in the expression of one or more characters. If you cross one individual which

[8] Wheldale, M., The Chemical Differentiation of Species, *Biochem. J.*, 5: 445–456 (1911); The Flower Pigments of *Antirrhinum Majus*. I. Method of Preparation, *ibid.*, 7: 87–91 (1913); Wheldale, M., and Bassett, H. L., The Flower Pigments of *Antirrhinum Majus*. II. The Pale Yellow or Ivory Pigment. III. The Red and Magenta Pigments, *ibid.*, 7: 441–444 (1913); 8: 204–208 (1914); The Chemical Interpretation of Some Mendelian Factors for Flower-Colour, *Proc. Roy. Soc.* (London), B, 87: 300–311 (1914); and Wheldale, M., Our Present Knowledge of the Chemistry of the Mendelian Factors for Flower-Colour, Pt. I and Pt. II, *J. Genetics*, 4: 109–129; 369–376 (1914 and 1915).

[9] Keeble, F., and Armstrong, E. F., The Oxydases of *Cytisus Adami*, *Proc. Roy. Soc.* (London), B, 85: 460–465 (1912); The Distribution of Oxydases in Plants and Their Role in the Formation of Pigments, *ibid.*, 85: 214–218 (1912); The Role of Oxydases in the Formation of the Anthocyan Pigments of Plants, *J. Genetics*, 2: 277–311 (1912); and Keeble, F., Armstrong, E. F., and Jones, W. N., The Formation of the Anthocyan Pigments of Plants, Pt. VI, *Proc. Roy. Soc.* (London), B, 87: 113–131 (1913).

is pure for one character with another which is pure for another character, the progeny will be intermediate in character between the two homozygous parents, and the F_1 generation would be heterozygous. The first-generation cross would contain the factors which are present in both parents. If these progeny are inbred (AB × AB), then you will get in the second generation a segregation of characters, AA, BA, AB, and BB, that is, four types. Theoretically the two types BA and AB are alike. Consequently you reproduce in the grandchildren the characters of the grandparents and likewise the characters of the parents.

For example, let us assume that A represents absence of color, and B represents a blue color. If the germ cells of one parent of a cross are all AA, *i.e.*, this individual has received no genetic factor for color from either of its parents, and the germ cells of the other parent of a cross are BB, *i.e.*, this individual has received a genetic factor for blue color from both of its parents, then we can predict the genetic constitution of the F_1 (first generation) and of the F_2 (second generation) offspring, which according to Mendel's law will have a genetic constitution as represented in the following diagram.



(Single Factor Mendelian Inheritance)

All the first-generation offspring will possess the blue pigment, inasmuch as each individual contains the factors which are present in both parents. When, however, the first-generation offspring are inbred, segregation will occur in the second generation, so that the F_2 offspring may possess any one of four possible genetic constitutions. (1) and (4) will breed true. Such individuals are called homozygous, *i.e.*, all the germ cells which they produce possess the same genetic constitution for the factor in question. The progeny produced by the self-fertilization of (1) will always be colorless, and the progeny produced by the self-fertilization of (4) will always be blue. (2) and (3) are heterozygous, *i.e.*, they produce germ cells which do not have the same genetic constitution for the factor in question, so that when AB or BA

are inbred resegregation will occur in the third generation to give a ratio of one colorless to four blue offspring, having respectively the genetic constitution of AA, AB, BA, and BB. Again (5) and (8) will
(5) (6) (7) (8)
be homozygous and will breed true, and (6) and (7) will again be heterozygous and give one colorless (albino), and three blue, only one of which (BB) will breed true.

Now assuming that color is the resultant of two factors, *e.g.*, an oxidizable chromogen C and an oxidizing enzyme E, in order to form color, both C and E must be present. We can thus postulate white flowers which have the following genetic formulas:

(1) White = AA = albino (contains neither chromogen nor oxidase).
(2) White = CC (contains only chromogen).
(3) White = EE (contains only enzyme).
(4) White = AC (contains only chromogen).
(5) White = AE (contains only enzyme).
(1), (2), and (3) are homozygous; (4) and (5) are heterozygous.

The following matings will produce

AA × CC = AC, white offspring (only one factor for color is present).
AA × EE = AE, white offspring (only one factor for color is present).
CC × EE = CE, *colored* offspring because *both* factors for color, enzyme and chromogen, are present.
or AC × AE = AA (colorless), AE (colorless), AC (colorless), and CE (colored), *i.e.*, one colored to three colorless offspring.

Such a postulate may serve to explain why certain white flowers crossed with other white flowers may yield color, whereas the same flowers self-fertilized yield only white offspring.

Still another form of white is known. *Here an inhibitor is present, some chemical reaction which prevents the formation of pigment when both enzyme and chromogen are present.* Such a white is known as a *dominant white* and, when crossed with a colored variety, results in white offspring. Thus, the white leghorn fowl is a *dominant white*, and yet it is not an albino. The white "Silky" fowl so far as the feathers are concerned is an albino (*recessive white*).

The animal pigment melanin is formed by the interaction of a chromogen and an oxidase. The oxidizing enzyme is apparently tyrosinase.[10] In the case of *Tenebrio molitor*, and probably rather gen-

[10] Gortner, R. A., Studies on Melanin. II. The Pigmentation of the Adult Periodical Cicada (*Tibicen septendecim* L.), *J. Biol. Chem.*, 10: 89–94 (1911); On Melanin, *Biochem. Bull.*, 1: 207–215 (1911); and Studies on Melanin. IV. The Origin of the Pigment and the Color Pattern in the Elytra of the Colorado Potato Beetle (*Leptinotarsa decemlineata* Say), *Am. Naturalist*, 45: 743–755 (1911).

erally in melanin formation, the chromogen appears to be dopa. *m*-Dihydroxyphenols (orcinol, resorcinol, and phloroglucinol) inhibit [11] the oxidation of tyrosine by tyrosinase, yet do not destroy the tyrosinase, nor are they themselves oxidized in preference. Apparently their presence is sufficient to inhibit the action. While no similar chemical has as yet been isolated from a dominant white organism, this test-tube experiment is at least suggestive of the mechanism which may be involved.

With the anthocyanins we do not always have a simple genetic formula, *i.e.*, a one-factor difference. Studies of the inheritance of color in the sweet pea have shown interesting results. The original wild sweet pea was in all probability a chocolate and purplish-blue flower. By breeding, various colors have been selected, and these can be recombined to the original color. Two whites may give purple, as already noted. This occurs in certain crosses of sweet-pea varieties. Red color is therefore due to two factors, A and B, and the loss of either produces a white flower. A third factor, R, is necessary to produce the blue color which when combined with red produces *purple*, but *R has no color when alone*, only when combined with A and B. Thus, flowers containing only A, B, AR, BR, or R are all colorless. Flowers containing AB are red, and flowers containing ABR are purple. From such data, *deductions* as to the *chemical* factors which are involved have been drawn.

Miss Wheldale (Mrs. Onslow) proposed a chemical interpretation of the function of factors for flower color in *Antirrhinum*. The factorial composition of the type of this species may be expressed by symbols as follows:

YYIILLTTDDBB

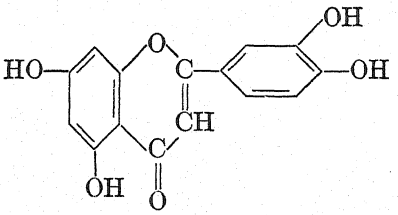
in which the symbols represent the following factors:

Y = a factor representing yellow color in the lips with ivory tube color; the absence of Y precludes the formation of any color in the flower.
I = a factor causing the production of ivory color in the tube and lips and inhibiting the formation of yellow pigment in the lips except on the palate.
L = a factor causing a magenta color in the lips.
T = a factor causing a magenta color in the tube.
D = a factor causing the production of a deepened or full color, changes a tinged ivory to magenta.
B = a factor converting a red anthocyanin to a magenta anthocyanin.

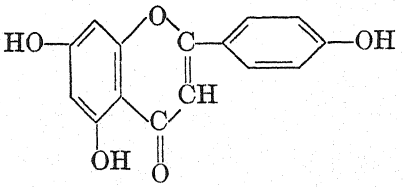
[11] Gortner, R. A., Studies on Melanin. III. The Inhibitory Action of Certain Phenolic Substances upon Tyrosinase. A Suggestion as to the Cause of Dominant and Recessive Whites, *J. Biol. Chem.*, 10: 113–122 (1911).

A study of the chemistry of the pigments involved and the mechanism of color production in *Antirrhinum* makes it possible to interpret these factors in chemical language. Thus, on a chemical basis they are:

Y = a factor causing the production of *luteolin* in the lips and of *apigenin* in the tube.



Luteolin



Apigenin

I = a factor causing the production of *apigenin* in the lips in place of luteolin (note that these two compounds differ only by one hydroxyl group).

L = a factor representing the presence of a reducing (?) or an oxidizing agent, enzyme (?), in the lips, which will produce an anthocyanin from the flavones when they are present.

T = a similar factor representing the presence of a reducing (?) or oxidizing (?) agent in the tube.

D = a factor, emulsin (?), causing the splitting off of the sugars from chains of linked glycosides and the addition or rearrangement of hydroxyl and methoxy groups attached to the rings of the flavone nucleus, thus causing additional anthocyanin to be formed by the hydrolysis of flavone glycosides.

B = a factor controlling the reaction (hydrogen-ion concentration) of the cell sap, thus shifting the color of a solution of the anthocyanin from red to magenta.

The following examples, which have been worked out for *Antirrhinum* varieties, show how these factors work: [12]

1. yyIILLTTDDBB — "white," lips and tube of corolla are pure white (no *flavone* is produced).
2. YYiilTTDDBB — "yellow," lips yellow, tube ivory [luteolin present but no oxidizing (or reducing) agent].
3. YYiiLLTTddbb — "yellow tinged with bronze," [luteolin present, oxidizing (or reducing) agent present].

[12] See Wheldale, *J. Genetics*, Vol. 4, color plate VII (1914), for an exact reproduction of these varieties.

4. YYIIllTTDDBB	"ivory," lips and tube ivory [luteolin absent, apigenin present, no oxidizing (or reducing) agent in lips].
5. YYiiLLTTDDbb	"bronze," [luteolin absent, apigenin present in lips, glycoside-splitting enzyme (?) present].
6. YYiiLLTTDDBB	"crimson," lips crimson, tube magenta, luteolin present in lips and tube, glycoside-splitting enzyme present, hydrogen-ion factor present, apigenin absent.
7. YYIILLTTDDBB	"magenta," all factors present producing deepest color.

We thus have hereditary factors which can be expressed by

1. The synthesis of definite chemical substances.
2. The modification of such substances once they are formed.
3. The control of enzyme action.
4. The modification of cell sap reaction.

The above discussion may be considered as only a suggestion of the possible aid which biochemistry may furnish in bringing about a solution of the problems of heredity. Only a bare beginning has been made in this important and fruitful field.

When anthocyanins are present, there is, as a rule, an excess of sugars in the leaves. Overton, in 1899, first called attention to the fact that there was more sugar in the red autumn leaves than in the normal green leaves. This has been confirmed by other workers who have also found more glycosides in the leaves containing anthocyanins. This is especially true for plants where the conducting systems have been injured so that the transport of carbohydrates is interfered with. A leaf which is injured may rapidly turn red, while the remainder of the plant has its full vigor. Since anthocyanin chromogens usually occur as glycosides and since the flavone nucleus probably comes originally from a carbohydrate source, it appears logical that an increased carbohydrate content should lead to the formation of excess chromogen which sooner or later is converted into an anthocyanin.

The artificial "feeding" of plants with sugars has resulted in many instances in anthocyanin formation. More intense light and low temperature produce anthocyanin, as has already been noted as a characteristic of alpine plants. Here we have high photosynthetic activity and a low requirement of carbohydrate utilization for energy (low enzyme activity for respiration, due to low temperature) both conducive to the formation of carbohydrates.

Mrs. Onslow [13] believes that chromogens acted on by oxidases pro-

[13] Onslow, M. Wheldale, The Anthocyanin Pigments of Plants, Second Edition, Revised, The University Press, Cambridge (1925).

duce anthocyanins, and she has produced good *genetical* evidence[14] for her theory. Keeble and Armstrong support her view. Everest, however, in his chemical studies, finds that flavones, when reduced, give colors corresponding to anthocyanins, and regards the anthocyanins as reduction products of the flavones. Willstätter's studies of the anthocyanins also indicate that they are reduction products of the flavones. Miss Wheldale has questioned the reduction theory, and the genetic data and certain of the biochemical data support her view. Perhaps the observation of Kozlowski[15] may be pertinent to the question. He finds that anthocyanins are reduced by sodium sulfite and sodium hydrosulfite to colorless compounds and that the color is regenerated by oxidation with iodine. He notes that Willstätter's red pigments formed by the *reduction* of flavonols are *not* decolorized by sulfites and that these red pigments of Willstätter are altered in color by elementary iodine. Kozlowski concludes that the hypothesis of Willstätter for the formation of anthocyanins by the reduction of flavonols is not justified and that we must return to the oxidation hypothesis.

The biochemical data are very well summed up by Armstrong,[16] as follows: "In general the distribution of pigments in flowers coincides exactly with that of oxydases. The oxydases, it is true, are more widely distributed than are the chromogens; but the distribution is in conformity with the oxydase-chromogen hypothesis, as will be illustrated by several typical examples, culled from the many available.

"The flowers of *Primula sinensis* and of *Dianthus barbatus* (Sweet William) show most epidermal oxydase in the most deeply coloured varieties, less in the less deeply coloured, and none at all in the white varieties. The white flowers of certain *Primula sinensis*, *Pisum sativum*, and *Lathyrus odoratus* have all been shown to contain oxydases, and the white colour is attributed to the absence of chromogen. In the Mont Blanc Star, the distribution of oxydase again parallels that of pigment. One flower had irregular magenta flaked petals with one exception, this particular petal being of a uniform magenta colour. The latter petal gave a well-marked oxydase reaction, the magenta patches on the others demonstrated a fair reaction, whilst the white portions did not respond to the test.

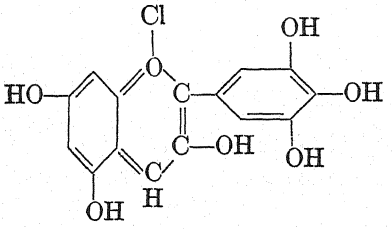
"Similarly, Sweet Williams were grown in full-coloured, white, and

[14] Wheldale, M., and Bassett, H. L., On a Supposed Synthesis of Anthocyanin, *J. Genetics*, 4: 103–107 (1914); Wheldale, M., On the Nature of Anthocyanin, *Proc. Cambridge Phil. Soc.*, 15: 137–168 (1909); and On the Formation of Anthocyanin, *J. Genetics*, 1: 133–158 (1911).

[15] Kozlowski, A., On the Reaction of Anthocyanins with the Sulfites, *Science*, 83: 465 (1936).

[16] Armstrong, E. F., Chemistry in the Twentieth Century, E. Benn, Ltd., London (1924).

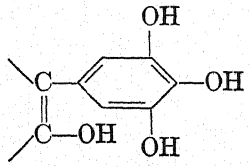
almost white varieties, the latter showing rosy dots or lines. The fully coloured flowers responded definitely to the tests for the presence of oxydases, whilst the white flowers also gave a definite but limited reaction—the white colour being probably due, as explained above, to the absence of chromogen. The white flowers with rosy dots showed oxydases only in the parts of the petals corresponding to the pigmented dots."



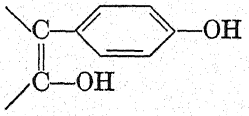
Delphinidin chloride

Delphinidin is a violet to blue-black anthocyanin, obtained from grapes, hollyhocks, petunias, violets, etc. In most instances it occurs as the diglycoside, the sugar residues being apparently attached to the hydroxyl groups on carbon-3 and carbon-5. The violet, *Viola tricolor*, contains this pigment as a rhamnoglycoside. Inasmuch as all the anthocyanins may be regarded as substitution products of the same nucleus, it is unnecessary to repeat all of the groupings in order to show the structural formula of the various anthocyanins.

Representing delphinidin as



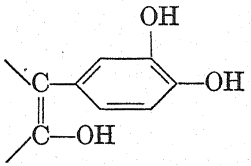
pelargonidin, the least oxygenated of any of the anthocyanins becomes



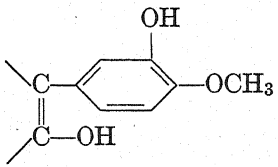
Pelargonidin occurs in many scarlet flowers, scarlet salvia, purple-red asters, etc., as the diglycoside, the residue being attached to the hydroxyl groups on carbon-3 and carbon-5. The diglycoside pelargo-

nin, along with several other related anthocyanin glyco. synthesized by Robinson.[17]

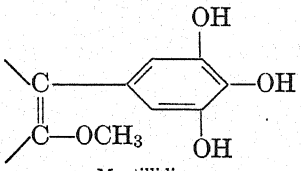
Cyanidin, the anthocyanin of deep red dahlias, cornflowe. fruits of cherries, cranberries, currants, mountain ash, etc., c. the grouping



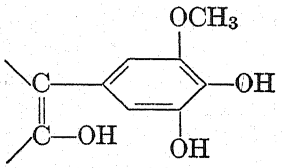
The anthocyanin colors are modified and the complexity of researches in this field are increased by the presence of ethoxy or methoxy groups in the molecule. Thus, *peonidin* is cyanidin monomethyl ether. Peonidin occurs in the deep violet-red peony, as a diglycoside.



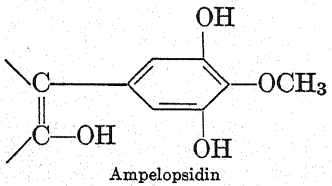
The problem of determining the formulas of mono- and dimethyl ethers of an anthocyanin is exceedingly difficult. Taking delphinidin as an example, there are five possible monomethyl ethers. Four have been found in nature. Formulas have been assigned to three of these.



Myrtillidin



Petunidin



Ampelopsidin

[17] Robinson, R., and Todd, A. R., Experiments on the Synthesis of Anthocyanins. XV. A Synthesis of Hirsutin Chloride, *J. Chem. Soc.*, 2293–2299 (1932); XVI. A Synthesis of Malvin Chloride, *ibid.*, 2299–2305 (1932); XVII. The Syntheses of Pelargonin, Peonin, and Cyanin Chlorides, *ibid.*, 2488–2496 (1932).

There are eleven possible dimethylethers. Only two have yet been isolated, and the formulas ascribed to these are only tentative.

Anderson *et al.*[18] find that an identical dark blue anthocyanin can be isolated from the skins of American grapes, *Vitis labrusca* (Concord), *Vistis aestivalis* (Norton), and *Vitis riparia* (Clinton), and that the compound is a monomethyl ether of delphinidin. The skin of the European dark blue grape, *Vitis vinifera,* contains the anthocyanin, oenidin, a dimethyl ether of delphinidin. Anderson[18] found that the hybrid grapes produced by crossing the American and European forms, are characterized by the presence of oenidin, the dimethoxy derivative. The tendency to produce a dimethoxy derivative of delphinidin is therefore a dominant in inheritance over the tendency to produce the monomethoxy derivative.

A number of our synthetic "coal-tar" dyes, *e.g.*, "rhodamine B" and tetramethylrosamine, are closely allied to the anthocyanin compounds.

Anthocyanin pigments often cause trouble in the canning industry on account of the changing in color of the fruit in the canning process or sometimes the actual perforation of the tin cans. In a study of this problem it was found[19] that the color change and the increased corrosion of the metal cans were due to the affinity of the anthocyanins for metals, *e.g.*, tin or iron. The metal salts are essentially insoluble and may precipitate on the inside of the can. The color of the metal salts is "dull" and "muddy," not bright as in the original fruit or juice. The color is shifted toward the violet end of the spectrum. "The reason why fruits of very low acid content but with large amounts of pigment, such as black cherries and blueberries, bring about more extensive corrosion of plain tin or earlier perforation of enameled tin than do more acid, less deeply colored fruits, such as red raspberries or sour cherries, is also clear. In the presence of large amounts of anthocyan, salts of tin with the acids of the fruits can have only momentary existence since they are immediately decomposed with transfer of the tin to combination with the anthocyan," and a liberation of the free acid to attack the metal again. This may continue until all the tin has been removed, the acid being used over and over again. With low anthocyanin and higher acid, the initial attack on the tin may be greater but it soon stops, for the anthocyanin is not present in sufficient amount to act as a reservoir for any large amount of tin.

[18] Anderson, R. J., A Contribution to the Chemistry of Grape Pigments, I. Concerning the Anthocyans in Norton and Concord Grapes, *J. Biol. Chem.*, 57: 795–813 (1923); (and Nabenhauer, F. P.), II. Concerning the Anthocyans in Clinton Grapes, *ibid.*, 61: 97–107 (1924); III. Concerning the Anthocyans in Seibel Grapes, *ibid.*, 61: 685–694 (1924); (and Nabenhauer, F. P.), IV. The Anthocyans in Isabella Grapes, *J. Am. Chem. Soc.*, 48: 2997–3003 (1926).

[19] Culpepper, C. W., and Caldwell, J. S., The Behavior of the Anthocyan Pigments in Canning, *J. Agr. Research*, 35: 107–132 (1927).

CHAPTER XXXIII

THE SIMPLE LIPIDS: THE FATTY ACIDS, GLYCERIDES, WAXES, STEROLS, AND BILE ACIDS

The natural fats and oils may be divided into two major groups, (1) the fatty or non-volatile fats and oils, and (2) the essential or volatile oils. Chemically the two groups are entirely distinct, group (1) being composed of esters of fatty acids (mainly glycerol esters), and group (2) being those plant products volatile with steam and separating as an oily layer in the distillate. Chemically the essential oils are aldehydes, alcohols, acids, hydrocarbons, terpenes, etc. Rarely are they the esters of the ordinary fatty acids. Oil of cloves, wintergreen, and turpentine are typical examples. Because of the great difference in chemical nature, the essential oils are not included in the term "lipids."

True fats are composed, in general, of only carbon, hydrogen, and oxygen, but certain derivatives or fat-like compounds, the "phospholipids," "lecithins," etc., contain nitrogen or phosphorus or both.

There is no generally adopted system for the classification of the lipids. The author believes, however, that essentially the classification proposed by Bloor[1] and adopted by Bull[2] as the basis of his classification is as satisfactory as any which has been proposed.

Bloor's classification, with minor modifications, is as follows:

Lipids

Substances having the following characteristics.

(*a*) Insolubility in water and solubility in the fat solvents, such as ether, chloroform, benzene.
(*b*) Relationship to the fatty acids as esters, either actual or potential.
(*c*) Utilization by living organisms.

I. Simple lipids—esters of fatty acids with various alcohols.
   (*a*) Fats—esters of the fatty acids with glycerol, solid at room temperature.

[1] Bloor, W. R., Biochemistry of the Fats, *Chem. Rev.*, 2: 243–300 (1925).
[2] Bull, H. B., The Biochemistry of the Lipids, John Wiley & Sons, New York (1937).

(*b*) Oils—esters of the fatty acids with glycerol, liquid at room temperature.

(*c*) Waxes—esters of the fatty acids with alcohols other than glycerol, alcohol commonly monatomic.

II. Compound lipids—esters of fatty acid-containing groups in addition to an alcohol and fatty acid.

(*a*) Phospholipids—substituted fats containing phosphoric acid and nitrogen—lecithin, cephalin, sphingomyelin.

(*b*) Cerebrosides—compounds of the fatty acids containing both a carbohydrate and a nitrogen base but containing neither glycerol nor phosphoric acid—phrenosin, kerasin, and nervon.

(*c*) Aminolipids.

(*d*) Sulfolipids.

III. Derived lipids—substances derived from the above groups by hydrolysis.

(*a*) Fatty acids.

(*b*) Sterols.

(*c*) Alcohols.

(*d*) Nitrogen bases.

A CLASSIFICATION OF THE FATTY ACIDS[3]

(Those which occur commonly are starred.)

I. The saturated fatty acids, acetic acid series, $C_nH_{2n}O_2$, $C_nH_{2n+1}COOH$ (only those with an even number of c-atoms are commonly found in natural fats).

Name	Carbon Atoms	Formula	Occurrence
A. With straight carbon chain.			
Acetic	2	$C_2H_4O_2$	Vinegar
Butyric	4	$C_4H_8O_2$	Milk fat
Caproic	6	$C_6H_{12}O_2$	Butter, cocoanut and palm nut oils, etc.
Caprylic	8	$C_8H_{16}O_2$	Cocoanut and palm nut oils, butter, etc.
Capric	10	$C_{10}H_{20}O_2$	Butter, cocoanut and palm nut oils, etc.
*Lauric	12	$C_{12}H_{24}O_2$	Laurel oil, spermaceti, etc.
*Myristic	14	$C_{14}H_{28}O_2$	In nutmeg butter
*Palmitic	16	$C_{16}H_{32}O_2$	Animal and vegetable fats
*Stearic	18	$C_{18}H_{36}O_2$	Animal and vegetable fats
*Arachidic	20	$C_{20}H_{40}O_2$	Peanut oil

[3] Many of the aliphatic acids occurring in plants or animal tissues, such as oxalic, tartaric, and citric, are not included, since they do not occur in fats or waxes.

A CLASSIFICATION OF THE FATTY ACIDS—*Continued*

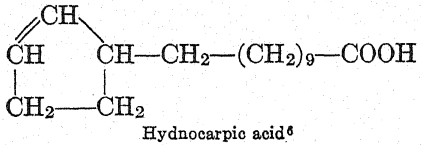
Name	Carbon Atoms	Formula	Occurrence
Behenic	22	$C_{22}H_{44}O_2$	Oil of ben, from seeds of *Moringa pterygosperma*
Lignoceric	24	$C_{24}H_{48}O_2$	In glucolipids and arachis oil
Carnaubic	24	$C_{24}H_{48}O_2$	Carnauba wax
Cerotic	26	$C_{26}H_{52}O_2$	Beeswax, Chinese wax, opium wax, wool fat, etc.
Melissic	30	$C_{30}H_{60}O_2$	
B. With branched carbon chain			
Tuberculo-stearic (10-Methyl stearic)	19	$C_{19}H_{38}O_2$	Human tubercle bacillus[4]
Phthioic	26	$C_{26}H_{52}O_2$	Human tubercle bacillus[5]
II. The unsaturated fatty acids			
1. Acrylic or oleic acid series		$C_nH_{2n-1}COOH$	
Tiglic		$C_5H_8O_2$	Croton Oil
Myristoleic		$C_{16}H_{30}O_2$	Fish and whale oils
Palmitoleic		$C_{16}H_{30}O_2$	Fish and whale oils
*Oleic		$C_{18}H_{34}O_2$	Animal and vegetable fats
Elaidic		$C_{18}H_{34}O_2$	Does not occur in natural fats
*Erucic		$C_{22}H_{42}O_2$	Rapeseed and similar oils
*Cetoleic		$C_{22}H_{42}O_2$	Marine animal and fish oils
2. Linoleic or linolic acid series		$C_nH_{2n-3}COOH$	
a. Acids with two double bonds			
*Linoleic		$C_{18}H_{32}O_2$	Vegetable oils, such as linseed, cottonseed, etc.
b. Acids with a triple-bond			
Tariric		$C_{18}H_{32}O_2$	Fats of *Picramnia* sp.
3. Linolenic acid series		$C_nH_{2n-5}COOH$	
Acids with three double bonds			
*Linolenic		$C_{18}H_{30}O_2$	Linseed oil
Elaeostearic		$C_{18}H_{30}O_2$	Chinese wood oil
4. Series		$C_nH_{2n-7}COOH$	
Acids with four double bonds			
*Clupanodonic		$C_{18}H_{28}O_2$	Japanese sardine oil
*Arachidonic		$C_{20}H_{32}O_2$	Lecithin, cephalin
III. Saturated monohydroxy acids		$C_nH_{2n}O_3$	
*Lactic		$C_3H_6O_3$	Lactic acid fermentation, carbohydrate metabolism

[4] Spielman, M. A., The Chemistry of the Lipids of Tubercle Bacilli XXXIX. The Constitution of Tuberculostearic Acid, *J. Biol. Chem.*, 106: 87–96 (1934).

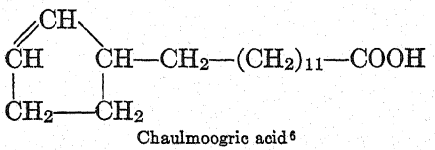
[5] Spielman, M. A., and Anderson, R. J., The Chemistry of the Lipids of Tubercle Bacilli XLII. Studies on Phthioic Acid, *J. Biol. Chem.*, 112: 759–767 (1936).

A CLASSIFICATION OF THE FATTY ACIDS—*Continued*

Name	Formula	Occurrence
β-Hydroxybutyric	$C_4H_8O_3$	Metabolism, animal
α-Hydroxy-*n*-decanic	$C_{10}H_{20}O_3$	Brain phospholipids
Sabinic	$C_{12}H_{24}O_3$	Conifer wax
Juniperic	$C_{16}H_{32}O_3$	Conifer wax
Lanopalmic	$C_{16}H_{32}O_3$	Wool fat
Cerebronic or phrenosinic	$C_{25}H_{50}O_3$	Phrenosin (brain tissue)
Cocceric	$C_{31}H_{62}O_3$	Cochineal wax
IV. Unsaturated monohydroxy acids	$C_nH_{2n-2}O_3$	
Ricinoleic	$C_{18}H_{34}O_3$	Castor oil
V. Saturated dihydroxy acids	$C_nH_{2n}O_4$	
Dihydroxystearic	$C_{18}H_{36}O_4$	Castor oil
Lanoceric	$C_{30}H_{60}O_4$	Wool fat
VI. Keto acids		
Pyruvic	$C_3H_4O_3$	Fermentation, metabolism intermediate
Acetoacetic	$C_4H_6O_3$	Metabolism intermediate
Levulinic	$C_5H_8O_3$	Carbohydrate metabolism
VII. Saturated dibasic acids	$C_nH_{2n-2}O_4$	
Thapsiac	$C_{18}H_{30}O_4$	Conifer wax
Japanic	$C_{22}H_{42}O_4$	Japan wax
VIII. Chaulmoogric series		
Cyclic acids with one double bond		
Hydnocarpic	$C_{16}H_{28}O_2$	Chaulmoogra oil
Chaulmoogric	$C_{18}H_{32}O_2$	Chaulmoogra oil



Hydnocarpic acid[6]

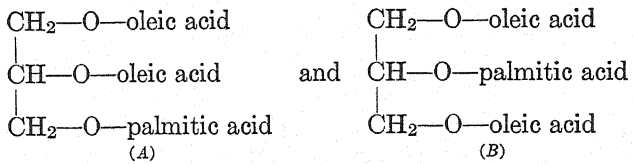


Chaulmoogric acid[6]

ESTERS OF GLYCEROL.—Inasmuch as glycerol is a trihydroxy alcohol, we have possible a number of esters. Thus, we may have monoglycerides, diglycerides, and triglycerides. In the case of the di- and triglycerides we may have the same or different fatty acids united in the ester, resulting in the formation of simple or mixed glycerides.

[6] Noller, C. R., and Adams, R., Synthesis of Dihydrochaulmoogric and Dihydrohydnocarpic acids. II., *J. Am. Chem. Soc.*, 48: 1080–1089 (1926); and Stanley, W. M., and Adams, R., The Synthesis of Chaulmoogric Acid from Hydnocarpic Acid, *ibid.*, 51: 1515–1518 (1929).

Mixed glycerides appear to be the general rule in nature. The milk fat of the monotreme or Australian spiny anteater is a notable exception, since it appears [7] to be pure triolein. When mixed glycerides occur, the problem of space relationships enters to complicate a study of the chemical constitution of the glyceride. Thus, for example, the two fats



will have the same empirical formula, will yield the same quantities of the same products upon hydrolysis, but because of the space configuration of the molecule, they will exhibit somewhat different physical properties. For example, (*A*) will contain an asymmetric carbon atom, whereas (*B*) will not. When such facts are kept in mind, together with the fact that the fats are relatively inert chemically and practically always occur as mixtures which are exceedingly difficult to separate, some appreciation can be gained of the difficulties involved in a study of the organic chemistry of the fats and oils.

Mono- and diglycerides apparently never occur in nature, nor are they present in freshly prepared oils and fats. Their presence in a fat or oil indicates that the material has undergone partial saponification. The presence of free fatty acid likewise indicates that hydrolysis of the neutral triglycerides has taken place.

SYNTHETIC GLYCERIDES.—King [8] notes that prior to 1929 seven different methods had been proposed for the synthesis of simple and mixed glycerides. Four of these general methods were devised by Grün, but the difficulty with most of these methods is that they result in an unpredictable rearrangement of the acyl groups which had been substituted in the glycerol molecule. Thus, for example, starting with the monohalogen-hydrin, the halogen may be on one of the α-hydroxyl groups of glycerol or on the β-hydroxyl group. It seems to be a

[7] Marston, H. R., The Milk of the Monotreme—*Echidna aculeata multiaculeata, Australian J. Exptl. Biol. Med. Sci.*, 3: 217–220 (1926).

[8] Averill, H. P., Roche, J. N., and King, C. G., Synthetic Glycerides. I. Preparation and Melting Points of Glycerides of Known Constitution, *J. Am. Chem. Soc.*, 51: 866–872 (1929); *cf.* also II. Refractive Indices of Glycerides of Known Constitution, *ibid.*, 52: 365–367 (1930); Robinson, H. E., Roche, J. N., and King, C. G., III. Mixed Triglycerides of the Distearin Series, *ibid.*, 54: 705–710 (1932); Jackson, D. T., and King, C. G., IV. Esters of Aromatic and Aliphatic Acids, *ibid.*, 55: 678–680 (1933); McElroy, O. E., and King, C. G., V. Mixed Triglycerides of the Dilaurin Series, *ibid.*, 56: 1191–1192 (1934); and Stimmel, B. F., Preparation and Properties of β-Monoglycerides, *ibid.*, 56: 1724–1725 (1934).

general rule that an acyl group substituted in the β-position on glycerol tends to wander to the more stable α-position, so that most of the compounds which have been described as β-monoglycerides actually have turned out to be α-monoglycerides owing to the wandering of the acyl group. Similarly most of the compounds which were earlier described as α-β-diglycerides have later turned out to be α-α-diglycerides, again because of the wandering of the group which had been substituted initially in the β-position. This problem of the wandering of the acyl groups has been stressed by several workers.[9]

Hill *et al.* studied the benzylidene derivatives of glycerol and isolated two isomeric benzylidene derivatives. The 1-3-benzylidene glycerol is crystalline and very stable. Here we have a six-membered ring. The 1-2-benzylidene derivative is a liquid, and its five-membered ring can rearrange to the 1-3-derivative. Using the 1-3-derivative, Hill *et al.* methylated the hydroxyl group in the β-position on the glycerol molecule and note that this β-methyl ether is the first recorded true β-derivative of glycerol where the position is absolutely certain.

Bergmann and Carter[10] used the 1-3-benzylidene glycerol to prepare β-monoglycerides and studied the properties of a number of these synthetic compounds. This appears to be one of the best methods by which the β-glyceride can be prepared. Even in this case unusual care must be taken not to heat the reaction mixture appreciably, particularly in the presence of acids, since wandering of the group from the β- to the α-position may take place in the presence of heat and acids.

In Bergmann's method glycerol is condensed with benzaldehyde to form the stable crystalline 1-3-benzylidene derivative. This compound is then treated in the presence of pyridine or quinoline with the acid chloride of the fatty acid which it is desired to esterify on the β-hydroxyl group, the pyridine or quinoline acting as a reservoir for the hydrochloric acid formed in the reaction. The benzylidene group is then removed by catalytic hydrogenation, leaving the β-monoglyceride as the residual product.

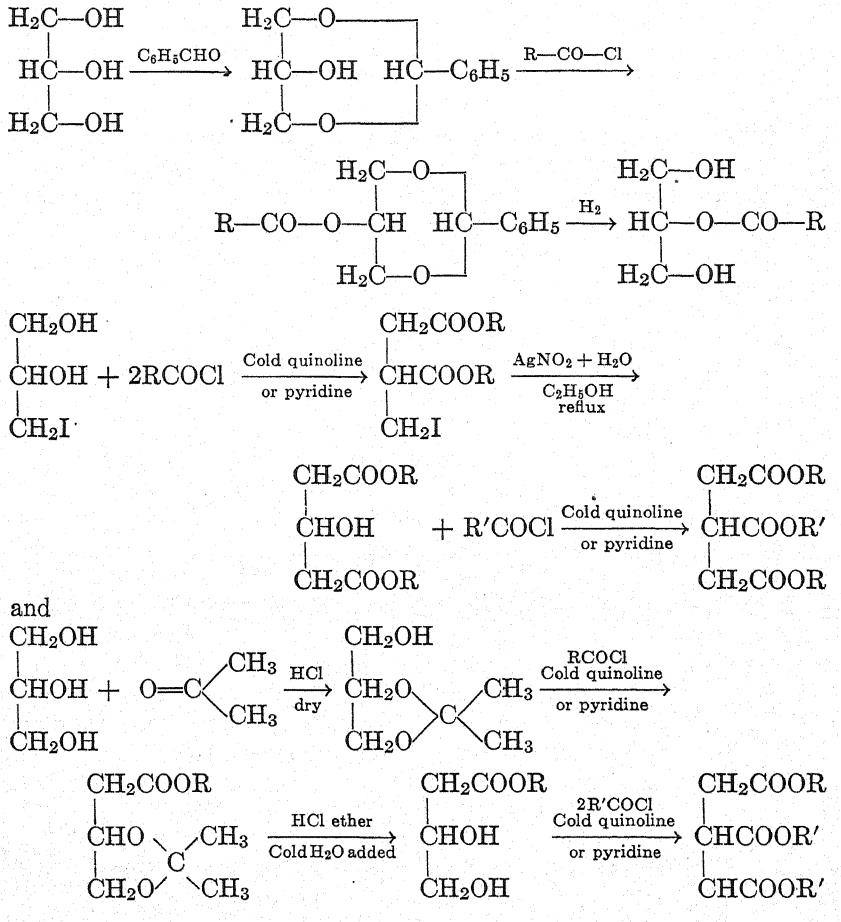
Shortly before his death in 1920, Fischer[11] devised methods for

[9] Hill, H. S., Whelen, M. S., and Hibbert, H., Studies on the Reactions Relating to Carbohydrates and Polysaccharides. XV. The Isomeric Benzylidene Glycerols, *J. Am. Chem. Soc.*, 50: 2235–2249 (1928); Hibbert, H., and Carter, N. M., Mechanism of Organic Reactions. I. The Wandering of Acyl Groups in Glycerol Esters, *ibid.*, 51: 1601–1613 (1929); and Fairbourne, A., and Cowdrey, G. W., The Partial Esterification of Polyhydric Alcohols. VII. The Unreliability of Proofs of the Structure of Disubstituted Glycerols, *J. Chem. Soc.*, 129–135 (1929).

[10] Bergmann, M., and Carter, N. M., Synthese von β-Glyceriden, *Z. physiol. Chem.*, 191: 211–221 (1930).

[11] Fischer, E., Bergmann, M., and Bärwind, H., Neue Synthese von α-Monoglyceriden, Ber., 53: 1589–1605 (1920).

the preparation of unsymmetrical triglycerides and also improved the methods for the synthesis of symmetrical di- and triglycerides. Fischer's methods, in combination with Bergmann's method noted above, are the ones generally used in modern glyceride synthesis. The reactions by the Fischer method can be indicated as follows:



It will be noted that in the first series of reactions using a halohydrin the first product formed is the α-β-diacyl derivative and that this rearranges when the halogen is removed to form the α-α-derivative. This more stable compound can then be treated with the acid chloride of a different fatty acid which will condense and add the acyl radical in the β-position, thus forming a symmetrical mixed triglyceride.

In the second series of reactions the resultant is an unsymmetrical mixed triglyceride. The α-β-acetone derivative of glycerol is formed, and only one acyl group is added to this in the α-position. Then, when the acetone derivative is saponified, a different acyl group can be added in the α-β-position.

The success of these two methods of synthesis lies in the fact that all the reactions can be carried out at temperatures not exceeding 35° C., excepting in that series of reactions where the $\beta \rightarrow \alpha$ shift is brought about. Low temperature and low acidity stabilize the β-compounds.

Using the above combination of reactions, a very large number of simple and mixed triglycerides have been prepared. The physical properties of these have been studied and compared with the physical properties of fractions isolated from natural fats or oils. In this way the structure of certain of the naturally occurring compounds has been definitely ascertained.

THE VOLATILE AND NON-VOLATILE FATTY ACIDS.—In the analysis of fats and oils the fatty acids are generally classified into the volatile and non-volatile groups. The fatty acids containing ten or less carbon atoms are classified as the *soluble fatty acids*. All fatty acids containing more than ten carbon atoms are classified as insoluble fatty acids, although lauric acid is slightly soluble in boiling water. As would be expected, the solubility of the fatty acids in water decreases as the number of carbon atoms increases. This has already been indicated in the discussion of the phenomena involved in the molecular orientation at interfaces. The *volatile fatty acids* are the same six fatty acids which are classified as soluble fatty acids. These are the only ones which can be distilled at atmospheric pressure. They possess a fairly high boiling point, but because of the high vapor tension they can be readily removed from an aqueous solution by steam distillation. Lauric acid is slightly volatile under the same conditions. Most natural fats contain some of the volatile fatty acids.

The titration value of a steam distillate of a saponified fat is the means of determining the *Reichert-Meissl number* of a fat, *i.e.*, the Reichert-Meissl number is the number of cc. of 0.1 *N* KOH required to neutralize the steam distillate obtained from 5 grams of saponified fats. Butterfat, because of its butyric acid content, has a very high (for fats) Reichert-Meissl number. Jensen[12] notes that this process accounts for 85 to 88 per cent of the total butyric acid, 85 to 100 per cent of the caproic acid, and 24 to 25 per cent of the caprylic acid present in the glycerides of butterfat. From 10 to 13.6 per cent of the fatty acids of butterfat are volatile.

[12] Jensen, O., Beiträge zur Kenntnis und Analyse der flüchtigen Fettsäuren in Palmfetten und Butter, *Z. Nahr. Genussm.*, 10: 265–283 (1905).

The insoluble or non-volatile fatty acids differ somewhat in chemical structure, which fact permits their classification into the saturated and unsaturated fatty acids, the unsaturated fatty acids being further subdivided according to the type and degree of unsaturation, as has already been noted in the early part of this chapter. Oleic, stearic, palmitic, linoleic, and linolenic acids constitute the principal insoluble fatty acids occurring in nature.

THE PROPERTIES OF NATURAL FATS AND OILS.—All the naturally occurring fats and oils have a specific gravity less than 1.0. Many of the fats can be obtained in a crystalline form. Both fats and oils are readily soluble in the so-called fat solvents, *i.e.*, ether, petroleum ether, chloroform, carbon bisulfide, and carbon tetrachloride, etc., but they are, in general, only sparingly soluble in alcohol, and because of this fact, ethyl alcohol is usually chosen as a solvent from which attempts are made to prepare crystalline fats. The fats themselves, the fatty acids derived from them, and especially the alkali salts of the fatty acids or soaps greatly reduce the surface tension of water. Accordingly, the soaps are used generally as emulsifying agents or detergents. The fats are excellent solvents for other compounds which have similar nature and which have similar solubilities. As we shall have occasion to note later, pure lard is often used as a solvent to gather the essence of flowers in the manufacture of perfumes. An analogous instance is the phenomenon which occurs when onions and butter are placed side by side in the icebox, or when cows eat wild garlic and the butterfat is tainted with the garlic.

THE STRUCTURE OF THE FATTY ACID MOLECULE.—Oleic acid is the most important of the unsaturated fatty acids, occurring as it does rather generally throughout the vegetable and animal kingdoms. The structure of oleic acid was established by the following steps:

1. An elementary analysis showed only carbon, hydrogen, and oxygen to be present in a proportion corresponding to the empirical formula $(C_9H_{17}O)_x$.

2. A molecular-weight determination showed that the formula must be $C_{18}H_{34}O_2$.

3. Esterification and titration with bases indicated the presence of a single carboxyl group.

4. The addition of iodine indicated the presence of one double bond, forming a diiodide (bromine could not be used, inasmuch as it substituted in the compound).

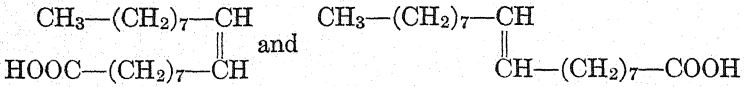
5. Stearic acid was obtained upon the reduction of oleic acid (the addition of two hydrogen atoms).

6. When oleic acid undergoes oxidation, a dihydroxystearic acid is first formed, the molecule then breaking at the double bond upon further oxidation into a saturated C_9 monobasic acid and a saturated

C_9 dibasic acid. Both these C_9 acids were found to be straight-chain carbon compounds. Therefore, the double bond of oleic acid was in the center of the carbon chain, and the formula could be written

$$CH_3(CH_2)_7—CH=CH(CH_2)_7—COOH$$

This, however, does not represent the true formula, for such a compound can exist in two stereomeric forms,



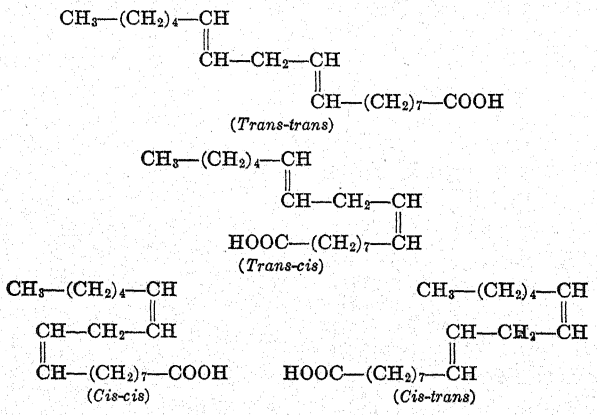
One of these is oleic acid (m.p. 14°); the other is elaidic acid (m.p. 45°). As already noted, elaidic acid does not occur in nature. Oleic acid is transformed into elaidic acid in the presence of a trace of NO_2.

The formation of stearic acid by the hydrogenation of oleic acid proves the formula of stearic acid.

It is relatively easy to determine the structure of the molecule of a fatty acid which contains only a single double bond. However, the determination of the structure of those fatty acids containing two or more double bonds is much more difficult. Linoleic acid *probably* has the formula

$$CH_3—(CH_2)_4—CH=CH—CH_2—CH=CH—(CH_2)_7—COOH$$

Such a formula has four possible *cis-trans* stereoisomers.[13]



Similarly, elostearic acid,

$$CH_3—(CH_2)_3—CH=CH—CH=CH—CH=CH—(CH_2)_7—COOH$$

[13] Nicolet, B. H., and Cox, H. L., C_{18} Acids. III. Four Tetrahydroxy-Stearic Acids Derived from Linolic Acid, and Their Significance with Regard to the Linolic Acid of Common Oils, *J. Am. Chem. Soc.*, 44: 144–152 (1922).

has eight possible stereomeric forms corresponding to those noted for linoleic acid. Elostearic acid occurs in Chinese wood oil,[14] from 90 to 95 per cent of the glycerides of Chinese wood oil being present as the esters of this acid.

The problem of *cis-trans* isomerism has taken on added significance since the study of Cooper and Edgar[15] of the comparative biological effects of *cis-trans* isomers. These workers studied maleic, fumaric, dibromosuccinic, succinic, citraconic, mesaconic, and itaconic acids. They found that the *trans*-acids were superior to their *cis*-isomerides in regard to (*a*) bactericidal action, (*b*) as protein precipitants, and (*c*) as "activating" enzymatic reactions. They found the *cis*-acids to be more strongly adsorbed by proteins than the *trans*-acids. They note that fumaric acid is a stronger disinfectant than phenol and is much less toxic to higher animals, and suggest that *trans*-derivatives may prove to be useful germicides.

The *cis*-acids and *trans*-acids show quite different behavior when their adsorption on charcoal is studied.[16] This adsorption appears to be apolar, *i.e.*, the undissociated molecule, and not the ions, is adsorbed.[17] When the equilibrium concentration was 0.05 mole per liter, the relative adsorbabilities were fumaric > mesaconic > succinic > citraconic > maleic > itaconic. At 0.10 mole per liter equilibrium concentration the order is fumaric > succinic > mesaconic > maleic = citraconic > itaconic. In an equilibrium solution saturated with the acids, the order of adsorbabilities is succinic > mesaconic > maleic > citraconic > itaconic > fumaric. The reason for the marked reversals in the saturated equilibrium solutions is explainable to a large degree by the solubilities of the acids, since fumaric acid has a solubility less than 1 per cent that of maleic acid and less than 10 per cent that of succinic acid. An even more striking difference in relative adsorbability of *cis-trans* isomers is afforded when one calculates the percentage of the amount in a saturated solution which is adsorbed by charcoal from that saturated solution. The amount of *trans*-fumaric acid adsorbed by charcoal in equilibrium with a saturated solution equaled 27 per cent of the amount of acid in solution. For the *cis*-maleic acid, the corresponding figure was only 0.27 per cent. For the

[14] Rhodes, F. H., and Welz, C. J., The Chemistry of Chinese Wood Oil, *Ind. Eng. Chem.*, 19: 68–73 (1927).

[15] Cooper, E. A., and Edgar, S. H., The Biological Significance of *Cis-Trans* Isomerism, *Biochem. J.*, 20: 1060–1070 (1926).

[16] Linner, E. R., and Gortner, R. A., Interfacial Energy and the Molecular Structure of Organic Compounds. III. The Effect of Organic Structure on Adsorbability, *J. Phys. Chem.*, 39: 35–67 (1935).

[17] Frampton, V. L., and Gortner, R. A., Electrokinetics. XVIII. Interfacial Energy and the Molecular Structure of Organic Compounds. IV. The Electrokinetic Behavior of Charcoals in Aqueous Solutions of Organic Acids, *J. Phys. Chem.*, 41: 567–582 (1937).

trans-mesaconic acid 6.98 per cent of the amount in a saturated solution is adsorbed at equilibrium, whereas with the *cis*-citraconic acid, only 0.09 per cent of the amount in solution is adsorbed at equilibrium.

THE HYDROGENATION OF OILS.—The pioneer work in this field was due to the activities of Sabatier,[18] who discovered that certain metals catalyzed the reaction $H_2 + R—CH=CH—R \rightarrow R—CH_2—CH_2—R$. The principal metals which have been employed are iron, cobalt, copper, nickel, platinum, and palladium. The reaction is carried out at an elevated temperature in the presence of a hydrogen atmosphere and the catalyst. By the means of catalytic hydrogenation, unsaturated hydrocarbons, such as ethylene, acetylene, etc., may be hydrogenated to the C_nH_{2n+2} series. Benzene is reduced to cyclohexane. The monosaccharides are readily reduced to the corresponding alcohols, etc.

An excellent history of the commercial application of the process of hydrogenation to oils is presented in the court decision of the case of Procter and Gamble Company *versus* Berlin Mills Company[19] (*cf.* also Richardson[20]). The lard substitutes, such as "Crisco," "Snowdrift," etc., do not represent anywhere nearly a complete hydrogenation of the vegetable oils. If the vegetable oils were completely hydrogenated, the resulting product would be brittle and similar to stearin or tallow. "Crisco" contains 20 to 25 per cent of saturated fats, 65 to 75 per cent of oleins, and 5 to 10 per cent of linoleins.

Selective hydrogenation can be utilized as an aid in the analysis of a fat. Thus, Hilditch and Stainsby[21] studied the hydrogenation of the body fat of the pig, following the degree of hydrogenation with the iodine number of the fat. Certain of their data are reproduced in Table LXXIX. It will be noted that in the original fat having an iodine number of 63 there is 13.5 per cent of linoleic acid. When this fat is hydrogenated to an iodine number of 48.9, only 1 per cent of linoleic acid remains and the increase in oleic acid practically accounts for the loss in the linoleic acid fraction. The stearic acid at this stage has increased only a little more than 1 per cent. A further increase in hydrogenation causes linoleic acid to disappear completely and reduces sharply the oleic fraction with a correspond-

[18] Sabatier, P., How I Have Been Led to the Direct Hydrogenation Method by Metallic Catalysts, *Ind. Eng. Chem.*, 18: 1005–1008 (1926).

[19] Hydrogenation of Oils, *J. Ind. Eng. Chem.*, 9: 1146–1148 (1917).

[20] Richardson, A. S., Knuth, C. A., and Milligan, C. H., Heterogeneous Catalysis. I. Selective Action of Catalytic Nickel in Hydrogenation of Certain Vegetable Oils. II. Hydrogenation of Marine Oils, *Ind. Eng. Chem.*, 16: 519–522 (1924); 17: 80–83 (1925); Richardson, A. S., and Snoddy, A. O., III. Hydrogenation of Cottonseed Oil with Platinum, *ibid.*, 18: 570–571 (1926).

[21] Hilditch, T. P., and Stainsby, W. J., The Body Fats of the Pig. IV. Progressive Hydrogenation as an Aid in the Study of Glyceride Structure, *Biochem. J.*, 29: 90–99 (1935).

TABLE LXXIX

THE SELECTIVE EFFECT OF HYDROGENATION ON PIG FAT
(DATA OF HILDITCH AND STAINSBY)

Iodine Number of Fat	Fatty Acids in Fat				
	Palmitic, mole per cent	Stearic, mole per cent	Oleic, mole per cent	Linoleic, mole per cent	Myristic, mole per cent
63.0*.....	27.3	14.4	40.9	13.5	2.8
48.9	27.4	15.7	52.1	1.0	2.8
43.0	27.4	21.4	47.3	None	2.9
35.8	27.4	29.6	39.2	None	2.8
28.7	27.5	36.2	32.5	None	2.8
18.7	27.5	47.5	21.2	None	2.8
10.0	27.5	57.4	11.3	None	2.8

* Original fat.

ing increase in the stearic acid fraction. Further hydrogenation progressively causes the oleic acid fraction to decrease and the stearic acid fraction to increase. It is evident, therefore, that those fatty acids containing two double bonds are preferentially hydrogenated so far as one of the double bonds is concerned. Apparently the double bond farthest from the carboxyl group is the one that hydrogenates the most readily. Furthermore an unsaturated acid in the α-position on the glycerol molecule is preferentially hydrogenated over a similar unsaturated acid on the β-position.

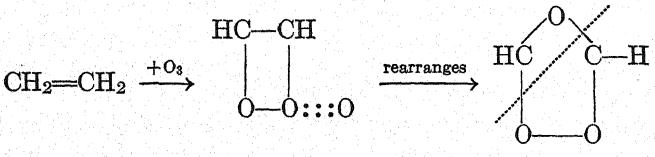
Waterman *et al.*[22] have particularly investigated conditions under which selective hydrogenation takes place. At high temperatures and low pressures and in the presence of a sluggish catalyst, there is a maximum of selective hydrogenation so far as the molecules of the fats and oils are concerned. Under these conditions a given iodine value will yield larger amounts of unsaturated fatty acids. High pressure and low temperature, on the contrary, yield more of the com-

[22] Waterman, H. I., and van Dijk, J. A., The Hardening of Linseed Oil at Very High Hydrogen Pressure, *Rec. trav. chim.*, 50: 679–680 (1931); Waterman, H. I., van Tussenbroek, M. J., and van Dijk, J. A., Influence of Pressure and Temperature on the Hardening of Soya-bean Oil, *ibid.*, 50: 793–795 (1931); Waterman, H. I., and van Dijk, J. A., Investigation on the Procedure of the Hardening of Linseed Oil at Very High Hydrogen-Pressure, *ibid.*, 50: 279–282 (1931); Waterman, H. I., and Zaayer, M., Hydrierung des Arachis-Öls, *ibid.*, 51: 401–406 (1932); Waterman, H. I., van Dijk, J. A., and van Vlodrop, C., The Effect of Varying Conditions in the Catalytic Hydrogenation of Fatty Oils on the Nature of the Reaction Product. I., *ibid.*, 51: 653–666 (1932); and Waterman, H. I., and van Vlodrop, C., II, *ibid.*, 52: 9–17 (1933).

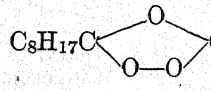
pletely saturated fatty acids. These relationships of temperature and pressure appear to be peculiar to the fatty acid molecules inasmuch as van Dijk *et al.*[23] found that, at high pressure and low temperature, palm oil could be hardened without destroying the carotenoids and that cod-liver oil could be hardened without destruction of vitamin D and without any great destruction of the compound which gives the color reaction with antimony trichloride and which is taken as a measure of vitamin A (*vide infra*). They note, however, that when tested biologically the vitamin-A content of the hydrogenated palm oil had been reduced.

The opposite of hydrogenation, *i.e.*, dehydrogenation, in which hydrogen is abstracted from the oils, has been more or less successfully accomplished on a small scale by heating the oils with catalysts. Dehydrogenation does not appear to have been practiced on a large commercial scale comparable to the hydrogenation industry. At least there is no general knowledge of such large-scale operation. If dehydrogenation on a large scale could be readily accomplished, the *drying oils* for paints could be readily made from the natural non-drying oils.

Ozonides.—Many organic compounds containing unsaturated linkages add ozone at the double bond. Thus, ethylene adds one molecule to ozone to form an ozonide,



When an ozonide is treated with water, it breaks up into aldehydes, ketones, or peroxides of aldehydes or ketones, and from a study of the resulting decomposition products, we can decide at what position the unsaturated double bond occurred in the original organic compound. Thus, oleic acid forms an ozonide in acetic acid solution, and on treatment of the oleic acid ozonide with water, it decomposes into hydrogen peroxide, pelargonic acid, and azelaic acid semialdehyde,



$$H_2O_2 + C_8H_{17}—CHO + CHO-(CH_2)_7—COOH$$

$$C_8H_{17}CHO + H_2O_2 = C_8H_{17}COOH + H_2O$$

This confirms the original structure proposed for oleic acid, *i.e.*, that the double bond is in the center of the molecule.

[23] van Dijk, J. A., Mees, R. T. A., and Waterman, H. I., Der Einfluss des Härtens auf die natürlichen Nebenbestandteile der Öle, *Biochem. Z.*, 245: 25–34 (1932).

SPONTANEOUS CHANGES IN FATS AND OILS.—RANCIDITY.—The term rancidity is used in two different industries to represent two entirely different changes which take place in fats and oils: (1) the hydrolysis of the glycerides, with the liberation of free fatty acids; and (2) the oxidation of fats and oils containing unsaturated acids, resulting in the formation of aldehydes, ketones, and acids, having a lower molecular weight than the acids which were naturally present. The term rancidity, as employed in the oil and fat industry, usually applies to the oxidative process, whereas in the dairy industry it applies to hydrolysis with the corresponding liberation of butyric acid which can be detected by its characteristic odor. The workers in the dairy indusdustry refer to the oxidative process as producing "tallowy butter" in contrast to the "rancid butter" produced by the hydrolytic process. As a general rule, oxidation and hydrolysis occur simultaneously, so that probably from the industrial standpoint no sharp line of demarcation can be drawn, although from the chemical standpoint the two mechanisms are sharply differentiated.

In the process of hydrolysis there is always an increase in titratable acidity. The presence of any trace of lipase (the fat-splitting enzyme) hastens and promotes this type of rancidity.

Oxygen is necessary in order to produce the oxidation type of rancidity. Heat, light, and moisture, together with the presence of certain metals which catalyze the reaction, hasten the oxidative process. Holm [24] notes that the greatest effect of light occurs when the light has a wave length of aproximately 3600 Å. Coe and LeClerc [25] state that in cottonseed oil and corn oil oxidative rancidity appears to be due primarily to the photochemical action of light on a compound which probably exists simultaneously in the oil or is produced from compounds which give rise to peroxides. Oils which were protected from light or which were exposed to light of wave length 4,900 to 5,800 Å remained free from rancidity even although they may have attained a peroxide value equal to or higher than oils which had been exposed to light and which had become exceedingly rancid.

The character of the fat and the environmental conditions determine whether one obtains aldehydes, acids, ketones, etc., so that an increased titration value need not occur, but usually does occur in oxidative rancidity. The Kreis color test (HCl+phloroglucinol + ether+the suspected fat) is the usual one for detecting deterioration due to oxidative rancidity, although a positive Kreis test in the crude oil may be due, not to decomposition products of the fat, but to sub-

[24] Greenbank, G. R., and Holm, G. E., Photochemical Oxidation of Cottonseed Oil, *Ind. Eng. Chem.*, 25: 167–168 (1933).

[25] Coe, M. R., and LeClerc, J. A., Photochemical Studies of Rancidity, *Ind. Eng. Chem.*, 26: 245–248 (1934).

stances derived from the seed from which the fat or oil was obtained.[26]

The Kreis color test is due to the presence of epihydrin aldehyde, $CH_2\overset{O}{\frown}CH\text{-}CHO$, and unless this aldehyde is present the product will not give a positive Kreis test. Accordingly the absence of color development in the Kreis test is not an invariable proof of the absence of rancidity, although Triebold,[27] using the spectrophotometer to measure the color intensity of the Kreis test, found a straight-line relationship between the logarithm of the reciprocal of the percentage of light transmission and the amount of oxygen absorbed per unit weight of the fat. This was true for a considerable variety of fats. Incidentally, this paper gives a very excellent résumé of the various theories concerning the mechanism of oxidative rancidity.

One feature of oxidative rancidity which contrasts this process sharply with the hydrolytic process is that the glycerol in oxidative rancidity disappears practically as fast as it is formed, being probably oxidized immediately on formation.

The strong odor characteristic of the lower fatty acids, especially of butyric acid, is readily produced in milk fat upon slight hydrolysis. Most other fats contain relatively small amounts of the lower fatty acids and much larger amounts of the higher fatty acids, stearic, palmitic, etc., which are almost odorless. Accordingly, hydrolysis of such fats produces comparatively little off-odor. The tallowy odor is produced by the oxidation of the unsaturated fatty acids and the formation of aldehydes and ketones. The oxidation of oleic acid is mainly responsible for the intense tallowy odor. Oxidation of linoleic acid produces less off-odors, while the oxidation of linolenic acid produces very slight amounts of off-odors. Milk fat accordingly contains the particular composition of fatty acids which makes possible an intense odor, resulting from either the hydrolytic or oxidative type of rancidity.[28]

The oxidation of fats and oils can be regarded as analogous to the oxidation of a great many systems, *e.g.*, the rubber industry, the petroleum industry, etc. One of the great advances in the rubber industry has been the finding of catalysts which inhibit the oxidation of rubber. Moureu and Dufraisse[29] were among the pioneers in the

[26] Smith, W. B., The Kreis Reaction of Cottonseed-Oil Products, *J. Ind. Eng. Chem.*, 12: 764–766 (1920).

[27] Triebold, H. O., Rancidity, *Cereal Chem.*, 8: 518–532 (1931).

[28] Holm, G. E., and Greenbank, G. R., Quantitative Aspects of the Kreis Test, *Ind. Eng. Chem.*, 15: 1051–1053 (1923); II. *ibid.*, 16: 518 (1924); Some Factors Concerned in the Autoxidation of Fats, *ibid.*, 16: 598–601 (1924); Measurement of Susceptibility of Fats to Oxidation, *ibid.*, 17: 625 (1925); The Keeping Quality of Butterfat, with Special Reference to Milk Powder, *Proc. World's Dairy Congress*, 2: 1253–1265 (1923); and Photochemical Oxidation of Cottonseed Oil, *Ind. Eng. Chem.*, 25: 167–168 (1933).

[29] Moureu, C., and Dufraisse, C., Catalysis and Auto-oxidation. Anti-oxygenic

study of antioxygenic catalysts. They note that one molecule of hydroquinone as an antioxidant can protect 40,000 molecules of acrolein from autoxidation, and they accordingly regard antioxidants as catalysts.

The mechanism which they propose for the reaction is that the oxidant A unites with oxygen to form a peroxide, AO_2. This peroxide oxidizes the antioxidant B with the formation of a peroxide, BO, at the same time the oxidant being transformed to a lower oxide, AO. AO and BO are antagonistic and mutually react to regenerate the three original molecules, A, B, and O_2 in their original state.

$$A + O_2 \rightarrow AO_2$$

$$AO_2 + B \rightarrow AO + BO$$

$$AO + BO \rightarrow A + B + O_2$$

Thus, the original molecule of the antioxidant is regenerated over and over again.

The oxidative process can be divided into two periods: (1) the period of induction, and (2) the period of active oxygen absorption. During the period of induction there is a negligible absorption of oxygen, and the susceptibility of a fat to oxidation may be determined by ascertaining under specified conditions the relative length of the induction period. Figure 158 shows the variation of the induction period, in terms of rate of oxygen absorption plotted against time, for several samples of lard. There was a marked correlation between the ease with which these various samples of fats became rancid when used in baked products and the shortness of the induction period.

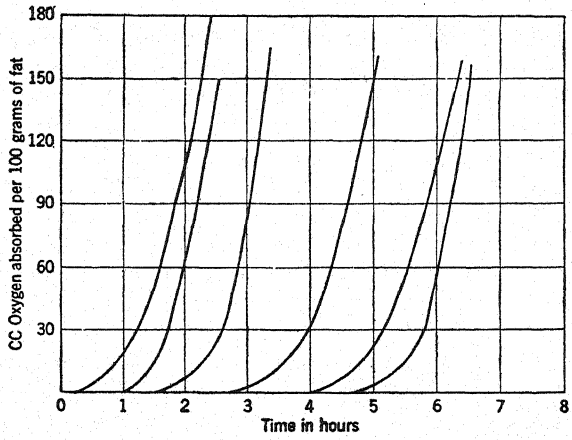


FIG. 158.—Showing the variation in the length of induction period of oxygen absorption for six commercial samples of lard. Temp. 95° C. (Data of Triebold.)[30]

and Pro-oxygenic Activity, *Chem. Rev.*, 3: 113–162 (1926); *cf.* also Berkman, S., Morrell, J. C., and Egloff, G., Inhibitors in Catalysis, Universal Oil Products Booklet No. 206, Universal Oil Products Company, Chicago (1936), 34 pp.

[30] Triebold, H. O., and Bailey, C. H., A Chemical Study of Rancidity. I. Autoxidation of Shortenings and Crackers, *Cereal Chem.*, 9: 50–64 (1932).

The form of the curves for oxygen absorption strongly suggests an autocatalytic reaction.

Mattill and Olcott and co-workers[31] have in recent years made very careful studies of the oxidative process with particular reference to the nature of the reaction and the chemical mechanism of the antioxidants. They agree with earlier workers that oxygen adds to the double bond of oleic acid to form what Staudinger calls a "moloxide" which can be defined as a peroxide compound of unknown composition and structure. They suggest that this moloxide rearranges to the more stable peroxide and that either the moloxide or the peroxide may react with water or the moloxide or peroxide compounds may break down of themselves to form aldehydes. The aldehydes so formed autoxidize to form acids with peracids as intermediate products. Therefore, in oxidative rancidity the original moloxides, the peroxides, and the reactive peracids are all involved in the oxidative mechanism. Mattill and Olcott, as well as many other workers, agree that autoxidation is a chain reaction which explains its apparent autocatalytic nature. They suggest that inhibitors act by breaking the chain and in particular by destroying the moloxides and peracids and that the inhibitors have little or no effect on the peroxides. This probably explains why determination of the amount of peroxides present does not conclusively measure either oxidative rancidity or the amount of oxygen which has been absorbed by fat. The chain reaction mechanism explains why a trace of an oxidized fat will catalyze oxidative rancidity in a large mass of neutral fat.

The chain reaction mechanism also explains the nature of the induction period,[32] and the high rate of reaction which follows the close of the induction period. During the induction period, the antioxidants or inhibitols are breaking the chains almost as fast as they are formed, but in this process they are themselves being used up or converted into other inactive products. When all the inhibitor has been transformed into inert products, the chains which form are no longer broken and the oxidative reaction proceeds at its normal but very high rate.

[31] Mattill, H. A., Antioxidants and the Autoxidation of Fats, *J. Biol. Chem.*, 90: 141–151 (1931); Olcott, H. S., II., *J. Am. Chem. Soc.*, 56: 2492–2493 (1934); French, R. B., Olcott, H. S., and Mattill, H. A., III. *Ind. Eng. Chem.*, 27: 724–728 (1935); Olcott, H. S., and Mattill, H. A., IV. Lecithin as an Antioxidant, *Oil and Soap*, 13: 98–100 (1936); Hamilton, L. A., and Olcott, H. S., V. Mode of Action of Anti- and Pro-oxidants, *ibid.*, 13: 127–129 (1936); Olcott, H. S., and Mattill, H. A., VI. Inhibitols, *J. Am. Chem. Soc.*, 58: 1627–1630 (1936); VII. Preliminary Classification of Inhibitors, *ibid.*, 58: 2204–2208 (1936); and Hamilton, L. A., and Olcott, H. S., VIII. Autoxidation of Oleic Acid, Methyl Oleate, and Oleyl Alcohol, *Ind. Eng. Chem.*, 29: 217–223 (1937).

[32] Stephens, H. N., Studies in Auto-oxidation. V. The Induction Period in Auto-oxidation, *J. Am. Chem. Soc.*, 58: 219–224 (1936).

Both Stephens, and Mattill and Olcott, agree that the inhibitor (or inhibitol) has been destroyed at the close of the induction period. Thus, it cannot be a true anticatalyst in the sense of Moureu and Dufraisse.

Mattill and Olcott studied the inhibiting effect of a great variety of organic compounds. They find that *o*- and *p*-diphenols are extremely effective as antioxidants. *m*-Diphenols, on the other hand, are inactive. In order to be effective, the hydroxyl groups must be on the benzene ring and not on aliphatic side chains. Inositol is completely ineffective. Only one hydroxyl group is required on the naphthalene nucleus. α-Naphthol is much more effective than β-naphthol. Quinone is a very effective antioxidant. β-Naphthoquinone is very effective. α-Naphthoquinone is essentially inert. Many of the vegetable oils contain a natural inhibitor which Mattill and Olcott have designated by the term inhibitol. This natural inhibitor occurs particularly in wheat germ and cottonseed oil. It can be concentrated in the non-saponifiable fraction by processes of crystallization and distillation similar to those used for the isolation and purification of vitamin E (*vide infra*). As yet it has not been separated from vitamin E, and the available evidence points to inhibitol and vitamin E as being compounds of very similar constitution, if they are not identical. Rather interestingly, the inhibitol concentrates are very potent antioxidants, so far as lard and other animal fats are concerned. They also protect purified fatty acids and fatty acid esters, but they do not protect the vegetable oils from which they were obtained.

In the last paper cited in the series, Olcott studied the autoxidation of oleic acid, methyl oleate, and oleyl alcohol. Under specific and carefully controlled conditions of oxygen absorption, he measured at various stages in the oxidation process the amount of oxygen absorbed, carbon dioxide and water evolved, the peroxides and aldehydes which were formed, the production of additional hydroxyl and carboxyl groups in the system being oxidized, the degree to which the ester was saponified, and the change in unsaturation as measured by change in iodine numbers. He found that the reactions were not identical for the three compounds studied. Oleyl alcohol absorbed approximately five atoms of oxygen per molecule, whereas oleic acid and methyl oleate absorbed only about four atoms. The peroxide level reached with methyl oleate and oleyl alcohol was approximately twice as great as with oleic acid. On the other hand, oleic acid formed from two to four times as many free hydroxyl groups as did methyl oleate or oleyl alcohol. Aldehydes were present from the beginning of the reaction but never reached any appreciable amount. They suggest that aldehydes are transient intermediate compounds being destroyed at approximately the same rate as they are formed. They suggest that possibly the difference between oleic acid, on the one hand, and

methyl oleate and oleyl alcohol, on the other, may be due to a reaction between a peroxide and the free carboxyl group of oleic acid whereby a hydroxyl group is formed and the peroxide is destroyed. Studies such as this, using pure compounds rather than the hopeless mixture of glycerides which characterizes the natural fats and oils, give promise of the eventual elucidation of the multiple reactions involved in the autoxidation of fats and oils.

Drying Oils.—Natural fats and oils exhibit two kinds of changes in the presence of air or oxygen. One type is the oxidative rancidity which we have just discussed. The other type is exhibited by the more highly unsaturated or drying oils which absorb oxygen and polymerize to stable insoluble films. These drying oils are valuable in the paint and varnish industry. The drying oils include particularly those in which the glycerides are at least in part esterified with acids belonging to the linoleic or linolenic series. Chief among the drying oils are tung oil, linseed oil, poppyseed oil, sunflower seed oil, hempseed oil, walnut oil, etc. Intermediate between the true drying oils and non-drying oils are a group of semi-drying oils including such oils as soybean oil and to a lesser degree corn oil, rapeseed oil, cottonseed oil, mustard oil, etc. These oils are more unsaturated than such non-drying oils as castor oil, olive oil, and peanut oil. In general, the iodine number of an oil may be taken as a probable indication of whether it is a drying oil, semi-drying oil, or a non-drying oil. The higher the iodine number, the greater is the probability that it will be a drying oil. However, the actual test for a drying oil is the exposure to air of a film of the oil on a glass plate. A drying oil should form a dry film which is insoluble in acetone in 2 to 6 days. A semi-drying oil will be somewhat sticky after even a week's exposure. A non-drying oil will still be fluid after 18 or 20 days of exposure to air.

As might be anticipated where such a vast industry as the oil and paint trade is concerned, there is an enormous literature with regard to the drying properties of oils. However, there is still no unanimity of opinion about what happens when a drying oil changes into an insoluble and resistant film. Either oxygen or heat or both are essential to the process. The modern view and the historical background are excellently summarized by Bradley,[33] who considers that polymerization is the essential process. The phenomena of polymerization and condensation were the subject of a general discussion before the

[33] Bradley, T. F., Drying Oils and Resins. Mechanism of the "Drying" Phenomenon, *Ind. Eng. Chem.*, 29: 440–445 (1937); and Drying Oils and Resins. Influence of Molecular Structure upon Oxygen and Heat Convertibility, *ibid.*, 29: 579–584 (1937).

Faraday Society in September, 1935.[34] While all the papers of this symposium are pertinent to the question of the transformations taking place in the drying of oils, the paper by Carothers [35] is particularly recommended. Carothers points out that polymerization and condensation are essentially identical. In the event that a compound possesses two functional groups, *e.g.*, a hydroxy acid containing both a hydroxyl and a carboxyl group, it can condense or polymerize by the condensation of the carboxyl group of one molecule with the hydroxyl group of another and under proper conditions this can continue almost indefinitely, resulting in the formation of a *linear polymer*, *i.e.*, a chain of condensed molecules which may reach a very great length. If the condensation were intramolecular, a lactone would be formed and the final product would be monomolecular. The intermolecular condensation should have at least as great a stability as lactones, and many lactones are exceedingly stable.

While polymers may result from the interaction of compounds containing only two functional groups, those compounds which contain polyfunctional groups are the ones which have the greatest industrial importance, and it is to polyfunctional molecules that we owe the behavior of such compounds as rubber, Bakelite, the vinyl and other synthetic resins, and in all probability the special properties of the drying oils. Both Carothers and Bradley stress the fact that the free fatty acids of linseed and tung oil do not show drying properties. The esters of these acids with monatomic alcohols do not show drying properties. The monoglycerides of these acids do not show drying properties. In general, the diglycerides of these acids do not show drying properties, although by heat treatment the diglycerides may acquire drying properties. During the heat treatment there is a loss of volatile constituents which may amount to as much as 10 per cent of the weight of the material, and Bradley believes that the heat treatment converts a considerable fraction of the diglycerides into triglycerides. The mono- and diglycol esters of the fatty acids of linseed and tung oils do not show drying properties prior to a heat treatment. It remains therefore for the triglycerides to be relatively specific in exhibiting the drying characteristics. Bradley applies Carothers' argument to explain this phenomenon. The esters of monatomic alcohols form only linear polymers. The esters of ethylene glycol have an increased number of functional groups but still not enough to form a stable three-dimensional polymeric structure.

[34] The Phenomena of Polymerisation and Condensation, A General Discussion held by the Faraday Society, September, 1935, *Trans. Faraday Soc.*, 32: 1–412 (1936).

[35] Carothers, W. H., Polymers and Polyfunctionality, *Trans. Faraday Soc.*, 32: 39–53 (1936).

The triglycerides have sufficient functional groups to form an interlocked three-dimensional polymer of exceedingly high molecular weight. Carothers points out that, in the example that we have already cited of a hydroxy acid condensing with itself, the bimolecular condensation product has caused the disappearance of half the initial functional groups. In the trimer 67 per cent of the functional groups have reacted, in the tetramer 75 per cent have reacted, etc. Taking p as the fraction of functional groups which react and x as the degree of polymerization, we have:

$$p = 1 - \frac{1}{x} \tag{185}$$

When $p = 0.8$, $x = 5$; when $p = 0.9$, $x = 10$; when $p = 0.95$, $x = 20$; $p = 0.99$, $x = 100$; when $p = 0.999$, $x = 1{,}000$. In other words, after p reaches approximately 0.95, a very slight increase in the functional groups reacting enormously increases the size of the resulting polymer, for the molecular weight has increased only 20 times at $p = 0.95$, but has increased 1,000 times at $p = 0.999$.

Carothers applies his reasoning to the vulcanization of rubber and states that a conservative estimate of the number of double bonds in the average rubber "molecule" is in the neighborhood of 5,000. Regarding these as active groups which can unite in some way with sulfur in the vulcanizing process, he calculates that all the rubber "molecules" would be linked together when only 0.04 per cent of the double bonds had disappeared or when the rubber had combined with only about 0.02 per cent of its weight of sulfur. Both Carothers and Bradley look upon polymerization as being due to the formation of actual valence bond cross-ties between the reacting molecules, although Bradley suggests that covalence forces may be assumed by those who wish to do so, provided that the covalence force postulated is of sufficient magnitude. It appears that in the particular fatty acids characteristic of the drying oils and in their particular arrangement relative to one another when they are esterified in the form of triglycerides, the various functional groups have acquired such space relationships as to favor intermolecular condensations and polymerizations, the resultant of which is the insoluble dry film which makes these triglycerides such valuable industrial products.

Saponification of Fats. The Formation of "Soaps."—Saponification of fats by alkalies brings about hydrolysis, with the formation of glycerol and the salts of the fatty acids. The salts of the higher fatty acids are soaps. The behavior of soaps as colloidal systems in themselves and as emulsifying agents has already been discussed under colloids.[36]

[36] For further references see: Fischer, Martin H., with the collaboration of McLaughlin, G. D., and Hooker, M. O., Soaps and Proteins, John Wiley & Sons,

Six general methods have been used to saponify fats:

1. The acidification process, in which the fat is heated with dilute sulfuric acid or hydrochloric acid under pressure at a temperature exceeding 100° C.

2. The autoclave process, in which the fats are treated with superheated steam in the presence of a small amount of calcium hydroxide.

3. Saponification with sodium hydroxide or potassium hydroxide solutions at the boiling temperature.

4. The cold saponification process, in which the required amount of concentrated alkali is added and the mixture is allowed to stand for several days.

5. The Twitchell process,[37] in which saponification is carried out in the presence of sulfobenzenestearic acid which acts as a catalyst.

6. The biological method, in which lipase or lipolytic enzymes are added to the fat, resulting in the formation of the free fatty acids and glycerol.

A discussion of the chemistry or technology of the soap manufacturing process lies outside of the scope of this volume. Here again there is a voluminous literature. Soaps have been known since almost the dawn of history. Perhaps, however, it is pertinent to make a mere mention of the very recent modern development which has resulted in the production of the products known commercially as "Gardinol" and to the housewife as "Dreft" and "Dreen." Someone has said that these products represent the only major change in the manufacture of detergents since the days of Cleopatra. These products are not soaps in the ordinary sense of the term but instead are the sodium salts of the sulfonic acids of the higher fatty alcohols, principally lauryl alcohol, cetyl alcohol, and octodecyl alcohol. The corresponding fatty acids are reduced to the alcohol by catalytic hydrogenation. The alcohols are then converted into the corresponding sulfonic acids. The sodium salts of these sulfonic acids possess the extremely desirable property of being marked surface-tension depressants, excellent detergents, and likewise possess the property of forming soluble salts with calcium and magnesium. Accordingly they

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New York (1921); McBain, J. W., Colloidal Chemistry of Soap, Pt. I. Solutions, Third Report on Colloid Chemistry, Brit. Assoc. Advancement Sci., pp. 2–31, London (1920); McBain, J. W., and Walls, E., Pt. II. The Soap Boiling Processes, Fourth Report on Colloid Chemistry, *ibid.*, pp. 244–263, London (1922); McBain, J. W., The States of Matter Exemplified by a Typical Colloid. Soap and the Soap Boiling Processes, Chap. V. of Colloid Chemistry, Theoretical and Applied, Vol. I, edited by Jerome Alexander, Chemical Catalog Company, New York (1926); and Nuttall, W. H., Industrial Application of Wetting Power, Fifth Report on Colloid Chemistry, Brit. Assoc. Advancement Sci., pp. 38–47 (1923).

[37] Perkin Medal Award to Ernst Twitchell, *J. Ind. Eng. Chem.*, 9: 192–198 (1917).

can be used as detergents in the hardest of water, even in saturated limewater. Furthermore the sulfonic acids are stronger acids than acetic acid, so that the sodium salts are not decomposed in weak solutions of the organic acids and exert their detergent properties even under acid conditions. Because of the high solubility of the salts of the alkaline earths of these products, they are readily rinsed from the fiber after the washing process, and because their solutions are not alkaline, there is less damage to wool and silk goods in the washing process than when somewhat alkaline soap solutions are used.

One of the sodium soaps of one of the fatty acids deserves particular mention in biochemical studies. While all the fatty-acid soaps possess bactericidal properties to a greater or a less degree, sodium ricinoleate possesses this property to a very high degree. Larson *et al.*[38] have particularly studied this problem. He finds that sodium ricinoleate has the property of detoxifying bacterial toxins and rendering more or less benign a very considerable number of pathogenic organisms. Carmichael[39] finds that sodium ricinoleate likewise detoxifies the toxalbumin ricin, so that when it is injected together with sodium ricinoleate as much as 2,000 lethal doses can be given without death ensuing. He also finds that it detoxifies rattlesnake venom.

We have noted that fats are hydrolyzed by lipase or lipolytic enzymes. In general, no marked specificity is exhibited by the lipases from various biological sources. However, the particular mixture of glycerides present in any given fat or oil does have a marked influence on the *rate* at which the various fats or oils are hydrolyzed by a given lipase. This is excellently demonstrated by the action of ricinus

[38] Larson, W. P., and Montank, I. A., The Effect of Wetting on the Pathogenicity and Viability of the Tubercle Bacillus, *Proc. Soc. Exptl. Biol. Med.*, 20: 229–232 (1923); Larson, W. P., and Nelson, E., The Effect of the Surface Tension of the Medium upon Bacterial Toxins, *ibid.*, 21: 278 (1923); Larson, W. P., Evans, R. D., and Nelson, E., The Effect of Sodium Ricinoleate upon Bacterial Toxins, and the Value of Soap-Toxin Mixtures as Antigens, *ibid.*, 22: 194–196 (1924); Larson, W. P., and Nelson, E., The Antigenic Properties of Pneumococci and Streptococci Treated with Sodium Ricinoleate, *ibid.*, 22: 357–358 (1924); Larson, W. P., and Colby, W., Immunization against Scarlet Fever Using Sodium Ricinoleate as a Detoxifying Agent, *ibid.*, 22: 549–550 (1924); Larson, W. P., and Halvorson, H. O., The Effect of Concentration upon the Neutralization of Toxin by Sodium Ricinoleate, *ibid.*, 22: 550–552 (1924); and Larson, W. P., Hancock, E. W., and Eder, H., Antidiphtheritic Immunization Using Sodium Ricinoleate as a Detoxifying Agent, *ibid.*, 22: 552–553 (1924); Larson, W. P., Halvorson, H. O., Evans, R. D., and Green, R. G., The Effect of Surface Tension Depressants upon Bacterial Toxins, *Colloid Symposium Monograph*, 3: 152–157 (1925).

[39] Carmichael, E. B., Detoxification of and the Immunity Production to Ricin by Sodium Ricinoleate, *Proc. Soc. Exptl. Biol. Med.*, 24: 5–7 (1926); and Detoxification of Rattlesnake Venom by Sodium Ricinoleate, *J. Pharmacol*, 31: 445–454 (1927).

lipase[40] on a series of fats and oils. The order of hydrolysis of the oils in this particular series of experiments was peanut > castor > corn > cottonseed > soybean > rape > olive > linseed > neat's foot > peach kernel > coconut > whale > fish > sperm. At the end of 10 hours, 97.2 per cent of the peanut oil had been hydrolyzed and only 19.5 per cent of the sperm oil.

GLYCEROL, THE ALCOHOL PRESENT IN FATS.—Glycerol is a triatomic alcohol, having the formula $CH_2OH—CHOH—CH_2OH$. Until recently the commercial source of glycerol has been the waste water of soap factories. Within the last few years, stimulated by the necessity of finding new sources of glycerol for the preparation of nitroglycerin, attention has been directed to the biological synthesis of this important substance. It has been prepared by bacterial and yeast fermentations of carbohydrates.[41]

In yeast fermentation, certain strains of yeast were found to be resistant to sodium sulfite. When they were added to solutions containing dextrose and sodium sulfite, alcoholic fermentation was largely inhibited, whereas glycerol fermentation was stimulated.

Glycerol is miscible in water in all proportions, and when strongly heated either alone or in the presence of a dehydrating agent, such as potassium bisulfate, it decomposes to form acrolein, $CH_2=CH—CHO$. Acrolein possesses a very penetrating, acrid odor. The formation of acrolein may be regarded as a test for the presence of glycerol. The odor of acrolein is noticeable when a tallow candle is blown out or when a fat is heated to too high a temperature.

The oxidation products of glycerol depend upon the oxidizing agent which is employed, *i.e.*, the oxidation potential. It may be oxidized to acrolein or to glyceric aldehyde, or completely oxidized to carbon dioxide and water. As one might expect, inasmuch as it is closely related to sugars, glycerol is relatively easily oxidized. Glycerol may be regarded as the alcohol which is formed by the reduction of the triose, glycerose. It shows the sugar character, inasmuch as it reduces many metallic salts, forms glycerolates with alkalies, is esterified by acids, *e.g.*, glycerol phosphoric acid ester (*vide infra*), and in fact has all the properties which might be expected to accompany a trihydroxy alcohol.

THE BIOLOGICAL SYNTHESIS OF FATS, AND THE FATTY ACIDS IN NUTRITION.—Although numerous theories have been evolved, there is little

[40] Longenecker, H. E., and Haley, D. E., Ricinus Lipase, Its Nature and Specificity, *J. Am. Chem. Soc.*, 57: 2019–2021 (1935).

[41] Anon., Chemistry of the Fermentation Process of Glycerine Production, *Chem. Age*, 28: 352–354 (1920); *cf.* also Barger, G., Enzymes and Fermentation, *Ann. Repts., Chem. Soc.*, London, 16: 166–170 (1919); Guilliermond, A., The Yeasts (translated and revised by Tanner, F. W.), John Wiley & Sons, New York (1920); and Connstein, W., and Lüdecke, K., Über Glycerin-Gewinnung durch Gärung, *Ber.*, 52B: 1385–1391 (1919).

certainty with regard to the mechanism whereby fats are synthesized by living organisms. The precursor appears to be, in general, carbohydrates and perhaps specifically glucose, and while proteins can give rise to fats in the animal body, it appears probable that this is brought about by first the formation of carbohydrate from protein and then the transformation of the carbohydrate into fat. At least, there is no evidence that those amino acids which cannot give rise to carbohydrate in the animal body are effective in the formation of fats.

Fischer suggested that perhaps three molecules of glucose might condense together and that this condensation product could then be reduced to form stearic acid or that two pentose molecules and one hexose molecule might condense to form palmitic acid, but there is no evidence that this is the actual mechanism, and the weight of evidence is wholly against this theory. In general, the theories which have received most consideration start with pyruvic acid, acetaldehyde, or aldol. Actually there is little difference as to which compound is regarded as a starting material, since acetaldehyde can arise by the decarboxylation of pyruvic acid, and aldol can arise by the condensation of two molecules of acetaldehyde. Both acetaldehyde and pyruvic acid are known breakdown products of carbohydrates. In any event, energy is necessary for the transformation of carbohydrate to fat, since the process involves the replacement of oxygen with hydrogen.

Until recently it has been assumed that the animal organism is capable of manufacturing, from carbohydrates, those fatty acids which the animal organism requires in its economy. However, in 1927, Evans and Burr [42] showed that the white rat on a diet highly purified and nearly devoid of fats exhibited growth abnormalities and delayed sexual development. Burr and his co-workers [43] have continued the study of the role of fat in the diet. They conclude that the white rat at least cannot synthesize adequate amounts of either linoleic or linolenic acid and that, in the absence of linoleic or linolenic acid from the diet, definite pathogenic phenomena occur. The skin becomes rough and scaly, metabolism is abnormal, sexual development is greatly delayed, water consumption is unusually high, reproduction either wholly fails or is abnormal, and lactation is deficient. Usually

[42] Evans, H. M., and Burr, G. O., New Dietary Deficiency with Highly Purified Diets. II. Supplementary Requirement of Diet of Pure Casein, Sucrose, and Salt, *Proc. Soc. Exptl. Biol. Med.*, 25: 41–49 (1927).

[43] Burr, G. O., and Burr, M. M., A New Deficiency Disease Produced by the Rigid Exclusion of Fat from the Diet, *J. Biol. Chem.*, 82: 345–367 (1929); Burr, G. O., Burr, M. M., and Brown, W. R., On the Nutritive Value of Certain Oils, *Proc. Soc. Exptl. Biol. Med.*, 28: 905–907 (1931); Burr, G. O., Burr, M. M., and Miller, E. S., On the Fatty Acids Essential in Nutrition, III, *J. Biol. Chem.*, 97: 1–9 (1932); Burr, G. O., and Brown, W. R., On the Fatty Acids Essential in Nutrition, *Proc. Soc. Exptl. Biol. Med.*, 30: 1349–1352 (1933).

the animal ceases growth when it has reached about 60–75 per cent of the adult weight, and the life span is reduced to approximately one-third of normal. Very small quantities of purified linoleic or linolenic acid or the esters of these acids are sufficient to cause the abnormal symptoms to disappear and normal growth to be resumed. Apparently linoleic and linolenic acids are specific and are interchangeable so far as this phenomenon is concerned.

As an extension of this series of studies, Hansen and Burr [44] demonstrated that in the normal adult rat the serum lipids had a decreased iodine number when the rat was on a fat-deficient diet, and Hansen and McQuarrie, in the Department of Pediatrics, at the University of Minnesota, have found that in many cases of infant eczema, the serum lipids are characterized by a lower iodine number than that of normal children. In such instances the low iodine numbers have been increased to more nearly the normal level by the feeding of vegetable oils high in linoleic or linolenic acid, *e.g.*, raw linseed oil or corn oil. In a number of instances, the inclusion of such oils in the diet has definitely improved the eczematous condition. From these studies it would appear as if man, as well as the white rat, cannot synthesize all the linoleic and linolenic acids which he needs.

THE ORIGIN OF MILK-FAT.—The synthesis of milk-fat has been studied by a great many investigators and probably is the easiest fat to study, since samples can be secured at frequent intervals without injury to the animal. Hilditch [45] has summarized our knowledge of the composition of butterfat with particular reference to the various sources from which the fatty acids characteristic of butterfat may be derived. One of the older theories was that the short-chain fatty acids of butterfat arose from the phospholipids of the blood. Hilditch rules out this theory and concludes that instead the milk-fats arise from the glycerides of the blood. He suggests that in the mammary gland there is the conversion of a part of the preformed oleoglycerides into glycerides containing the shorter-chained fatty acids, such as butyric and caproic, and that at the same time some of the oleic acid is converted into stearic acid. He suggests that this implies an enzymatic oxidation-reduction system operating from the alkyl end of the oleic acid group combined with the glycerol. He points out that such an enzymatic mechanism should be specific for C_{18}-fatty acids and should be blocked providing that the fatty acids available were largely of the C_{20}- or C_{22}-series. Cod-liver oil contains large proportions of

[44] Hansen, A. E., and Burr, G. O., Iodine Numbers of Serum Lipids in Rats Fed on Fat-Free Diets, *Proc. Soc. Exptl. Biol. Med.*, 30: 1201–1203 (1933).

[45] Hilditch, T. P., The Source of the Typical Components of Milk-Fats: A Hypothesis Suggested by Recent Work on their Glyceride Structure, *Compt.-rend.*, Vol. II, Ve Congrès International Technique et Chimique des Industries Agricoles, Scheveningue (1937), pp. 367–383.

the highly unsaturated acids of the C_{20}- and C_{22}-series. Accordingly Hilditch fed milch cows cod-liver oil in one series of experiments and linseed oil in another series, and analyzed the milk and total milk-fat, butter fatty acids, and the distribution of the individual fatty acids in the fat before, during, and after the feeding of the specific oils. Table LXXX shows certain of the results obtained. It will be noted

TABLE LXXX

EFFECT ON THE COMPOSITION OF MILK-FAT OF THE FEEDING OF COD-LIVER OIL AND LINSEED OIL
(DATA OF HILDITCH)

	Cod-liver Oil			Linseed Oil		
	Before	During	After 2 Wks.	Before	During	After 2 Wks.
Total milk-fat per day, lb.	2.22	1.30	2.04	2.80	2.46	2.74
Butter acids per day, lb...	2.10	1.24	1.95	2.66	2.33	2.60
Fatty Acids	Percentage Distribution of Fatty Acids in Butter Acids					
Butyric	4.28	1.61	4.62	4.51	4.29	4.23
Caproic	1.90	0.81	2.05	1.88	2.15	1.92
Caprylic	2.38	0.81	2.05	1.88	1.29	1.92
Capric	2.86	1.61	3.08	3.00	2.15	3.07
Lauric	3.81	4.03	3.59	3.76	3.00	3.85
Myristic	10.0	6.45	10.26	10.15	8.15	10.0
Palmitic	25.24	23.39	25.13	25.19	21.89	25.38
Stearic	12.38	8.06	12.31	12.41	9.87	12.31
Arachidic	0.95	0.81	0.51	0.75	0.43	0.77
Oleic	30.95	42.74	30.76	30.81	39.48	31.15
Octadecadienoic	4.28	4.83	4.61	4.51	6.00	4.23
C_{20}-C_{22}	0.95	4.83	1.03	1.13	1.29	1.15

that the feeding of the cod-liver oil containing the C_{20}- and C_{22}-series of unsaturated fatty acids did, in a very appreciable degree, block the synthesis of butyric, caproic, caprylic, and capric acids. On the other hand, the C_{20}- and C_{22}-acids appeared in the butterfat in much larger quantities than normal. Hilditch notes that only 0.02 pound per day of butyric acid appeared in the milk-fat during the cod-liver oil feeding in contrast to the normal of approximately 0.09 pound.

THE DIGESTION AND TRANSPORT OF FATS.—Bloor [46] has pointed out that fats differ sharply from proteins and carbohydrates in that neither

[46] Bloor, W. R., Lipid Metabolism, Chapter XXI of Harrow and Sherwin's Textbook of Biochemistry, W. B. Saunders Company, Philadelphia (1935).

the fats nor the fatty acids are water-soluble, and accordingly they are transported and metabolized in the animal body by other mechanisms than simply aqueous diffusion.

In the intestinal tract the fats are completely emulsified, the emulsifying agent being largely soaps which are formed from free fatty acids and the alkali of the bile and pancreatic secretions. Lipase then attacks the emulsified fat and hydrolyzes it into its constituent fatty acids and glycerol. The bile salts have the power to "dissolve" the free fatty acids or the fatty acid soaps and bring them into a form which is freely diffusible into the intestinal mucosa. They are thus able to pass the epithelial walls and be absorbed into the epithelial cells of the mucous membrane. Here they are apparently resynthesized into fats. The mechanism of this resynthesis is unknown, although Sinclair [47] suggests that the phospholipids of the intestinal mucosa are the active agents, or at least are involved in the synthetic process. His experiments suggest that the fatty acids first combine with the phospholipids, and the neutral fats are split off, and the phospholipid is regenerated.

In contrast to amino acids and the simple sugars, the fat does not go directly through the intestinal mucosa into the blood stream but instead passes largely into the lymph circulation and through the thoracic duct into the venous circulation. It therefore does not pass first through the liver but rather short-circuits the liver and does not return to the liver except through the arterial circulation. In the blood stream the fats are present in the form of an extremely finely divided emulsion which has been called the chylomicron [48] emulsion, the chylomicrons ranging from 0.5 to 1.0 μ in diameter. The chylomicron emulsion increases enormously after the ingestion of fat and then gradually falls through a period of hours to the normal level.

The question as to how fat is removed from the blood and utilized by the various cells and tissues is even more obscure than are some of the problems relating to its transport into and across the intestinal mucosa. There is no evidence at the present time that lipase hydrolysis is involved during this process, and there is great hope that "tagged molecules" of the fatty acids containing deuterium or other isotopes may enable the biochemist to follow the movement of specific molecules across the membranes of the intestinal mucosa, and then to trace their path in the lymph stream either to their ultimate depots in the body or to the places where they are burned for energy, and thus elucidate the biological mechanisms involved.

[47] Sinclair, R. G., The Role of the Phospholipids of the Intestinal Mucosa in Fat Absorption, *J. Biol. Chem.*, 82: 117–136 (1929).

[48] Gage, S. H., and Fish, P. A., Fat Digestion, Absorption, and Assimilation in Man and Animals as Determined by the Dark Field Microscope, and a Fat-Soluble Dye, *Am. J. Anat.*, 34: 1–85 (1924).

THE WAXES

Waxes differ from fats in that they are esters of mono- (or in some instances di-) hydroxy saturated alcohols or of sterols with certain of the higher fatty acids. Certain of the higher alcohols of the $C_nH_{2n+2}O$ series which occur in waxes are shown in Table LXXXI. Most of

TABLE LXXXI

HIGHER ALCOHOLS OF THE C_nH_{2n+2} SERIES OCCURRING IN WAXES

Compound	Empirical Formula	Occurrence
Dodecanol	$C_{12}H_{26}O$	Cuticle wax of *Cascara sagrada*
Cetyl alcohol	$C_{16}H_{34}O$	Spermaceti (as palmitate)
Octodecyl alcohol	$C_{18}H_{38}O$	Spermaceti (as palmitate)
Carnaubyl alcohol	$C_{24}H_{50}O$	Wool fat
Neoceryl alcohol	$C_{25}H_{52}O$	Beeswax (*Apis mellifera*)
Ceryl alcohol	$C_{26}H_{54}O$	Beeswax, wool fat, carnauba wax
n-Hexacosanol	$C_{26}H_{54}O$	Cocksfoot (*Dactylis glomerata*), cuticle wax
Dimyristyl carbinol	$C_{27}H_{56}O$	Apple cuticle wax
n-Octacosanol	$C_{28}H_{58}O$	Apple cuticle wax
Montan alcohol	$C_{29}H_{60}O$	Beeswax
Myricyl alcohol	$C_{30}H_{62}O$	Carnauba wax as cerotate, beeswax as palmitate
Cocceryl alcohol	$C_{30}H_{62}O_2$	Cochineal wax (*Coccus cacti*)
Melissyl alcohol	$C_{31}H_{64}O$	Beeswax
Laccerol	$C_{32}H_{66}O$	Lac wax (*Coccus lacca*)
Incarnatyl alcohol	$C_{34}H_{70}O$	Beeswax, clover wax, especially *Trifolium incarnatum*

the alcohols shown in this table occur in nature only in the waxes. The waxes occur as insect secretions and as protective coatings on the cuticle of the leaves or fruits of plants. They rarely occur as cell constituents. The plant waxes often occur in association with hydrocarbons of the paraffin series, and Channon and Chibnall [49] suggest that the paraffins arise from a reduction of a ketone which in turn has been formed by the condensation of two molecules of a fatty acid. Thus, hentricontane, $C_{31}H_{64}$, has been found to occur rather commonly in nature, and this might arise [50] from palmitic acid which condenses to the ketone and then is reduced to the paraffin. However,

[49] Channon, H. J., and Chibnall, A. C., The Ether-Soluble Substances of Cabbage Leaf Cytoplasm. V. The Isolation of *n*-Nonacosane and Di-*n*-tetradecyl Ketone, *Biochem. J.*, 23: 168–175 (1929).

[50] Collison, D. L., and Smedley-Maclean, Ida, The Nature of the Lipoid Matter Extracted from Green Leaves (Spinach and Cabbage), *Biochem. J.*, 25: 606–613 (1931).

Chibnall and Piper [51] note that the paraffins are nearly always accompanied by fatty acids containing approximately the same number of carbon atoms and that no one has as yet identified the natural occurrence of the ketone which is postulated under the fatty acid condensation theory. Liver oils frequently contain rather large amounts of hydrocarbons. This is notably true of the shark-liver oils.

Waxes are much more difficultly saponified than are those fats and oils which are esters of glycerol. As a rule, saponification of waxes requires rather long boiling with alcoholic potassium hydroxide. The fatty acids of the waxes, of course, pass into the alkali as "soaps," but the higher alcohols are not water-soluble and appear in the "unsaponifiable matter" fraction. Thus, whereas the unsaponifiable matter of a true fat may amount to only 1 or 2 per cent of the material taken, the corresponding fraction for the liquid waxes may range from 31 to 43 per cent, and for the solid waxes may reach as much as 55 per cent of the material which was saponified. Chibnall *et al.*[52] have studied the wax constituents of apple cuticle and of certain of the grasses. These papers may be consulted for some of the more modern technics which can be applied in studies of this type.

THE STEROLS

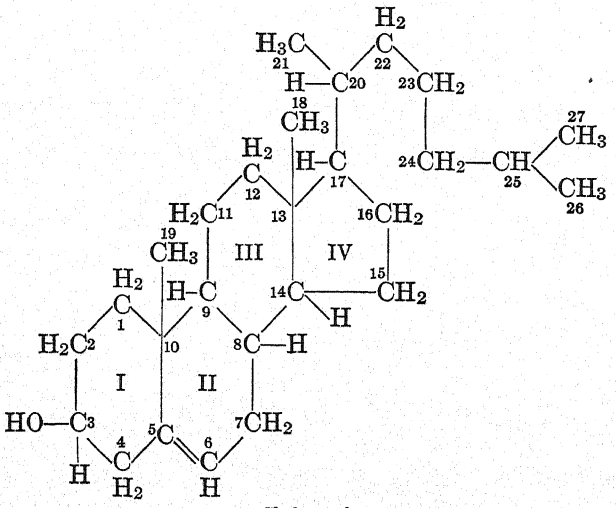
In discussing the saponins we have already indicated that those compounds, as well as the toad poisons, the sex hormones, and the sterols, are derivatives of the cyclopentenophenanthrene nucleus. The structure of the sterols has been a very active subject of investigation for many years, but it was in the period 1932–1934 that the structure of cholesterol was finally ascertained.

The name sterol is derived from the Greek *stereos*, meaning solid, with the *ol* ending, and therefore means literally a solid alcohol. The prefix, Greek, *chole*, means bile, and therefore cholesterol means literally the solid alcohol derived from bile. Cholesterol has been known for many years as the most common constituent of human gallstones. It is present in all cells of the animal organism and in the higher animals occurs in large amounts in the brain and nerve tissues. It is also present in very appreciable amounts in egg yolk and in the suprarenal gland. Cholesterol contains one hydroxyl group on carbon-3 and one double bond between carbon-5 and carbon-6. It occurs in animal tissues almost invariably in association with the hydrogenated com-

[51] Chibnall, A. C., and Piper, S. H., The Metabolism of Plant and Insect Waxes, *Biochem. J.*, 28: 2209–2219 (1934).

[52] Chibnall, A. C., Piper, S. H., Pollard, A., Smith, J. A. B., and Williams, E. F., The Wax Constituents of the Apple Cuticle, *Biochem. J.*, 25: 2095–2110 (1931); and Pollard, A., Chibnall, A. C., and Piper, S. H., The Wax Constituents of Forage Grasses. I. Cocksfoot and Perennial Ryegrass, *ibid.*, 25: 2111–2122 (1931).

pound, dihydrocholesterol, where the double bond between carbon-5 and carbon-6 has been saturated. In animal feces there is excreted a sterol known as coprosterol, from the Greek *kopro*, meaning dung. Coprosterol is a dihydrocholesterol isomeric with the dihydrocholesterol occurring in normal cells and tissues. Apparently the isomerism is on carbon-5 of the sterol nucleus. It will be noted that, if the double bond between carbon-5 and carbon-6 becomes hydrogenated, carbon-5 becomes an asymmetric carbon atom. There is thus the possibility of a *cis-trans*-isomerism revolving around the groups on carbon-5. The —OH group at carbon-3 is not involved in this particular isomerism, since when cholesterol is reduced to the hydrocarbon, *cholestane* (CH_2 at carbon-3 and the double bond at carbon-5 and carbon-6 saturated), a different hydrocarbon is obtained from that which results from the reduction of coprosterol. The hydrocarbon from coprosterol is known as *coprostane* and is isomeric with cholestane. In cholestane ring I and ring II correspond to a *trans*-decalin. In coprostane we have *cis*-decalin. Another way of expressing this is that in cholestane the hydrogen on carbon-5 is directed back of the plane of the diagram, whereas in coprostane the hydrogen is directed in the "near" position.



Cholesterol

Cholesterol can be conveniently prepared from sheep or hog brains. These should be macerated and mixed with anhydrous calcium sulfate (plaster of Paris) in sufficient quantities so that, when the plaster of Paris has been converted to gypsum, the water present in the biological material will be bound as water of crystallization. The mass of

brain tissue containing the gypsum is then powdered and extracted with ether. Crude cholesterol dissolves and can be crystallized directly by concentrating the ether extract, or the ether extract can be saponified and the cholesterol extracted and recrystallized from the non-saponifiable residue.

A series of color reactions is produced by the sterols when they are treated with strong acids under dehydrating conditions. In the *Liebermann* or *Liebermann-Burchard reaction* the sterol is treated with chloroform, acetic anhydride, and concentrated sulfuric acid. The color produced is a beautiful violet. In the *Salkowski reaction* a solution of the sterol in chloroform is shaken with concentrated sulfuric acid. A beautiful cherry-red color appears in both layers. The *Steinle-Kahlenberg reaction* produces a clear purple solution changing to cobalt blue on exposure to light when the sterols-in-chloroform solution is treated with antimony pentachloride. In the *Tschugajeff reaction* a glacial acetic acid solution of the sterol is boiled after the addition of zinc chloride and acetyl chloride. Whitby [53] and Schoenheimer, Dam, and von Gottberg [54] have outlined the use of the color reactions for the colorimetric estimation of the sterols.

The color reactions are apparently specific for the unsaturated sterols, for they are not given by dihydrocholesterol, coprosterol, or the hydrocarbons derived from the sterols. The *Rosenheim reaction*, where the sterol is dissolved in chloroform and a few drops of a strong aqueous solution of trichloracetic acid is added, appears to be more or less specific for ergosterol and for sterols having a double bond between carbon-4 and carbon-5. Bloor's [55] colorimetric method is an adaptation of the Liebermann-Burchard reaction.

Cholesterol may be estimated gravimetrically [56] as the cholesteride of digitonin inasmuch as cholesterol forms an insoluble precipitate with digitonin as has already been noted.

Cholesterol inhibits the reaction of saponins and prevents the hemolysis of red cells in the presence of saponins.[57] The esters of cholesterol do not inhibit the action of saponins. It thus appears probable that cholesterol forms a definite compound with all saponins

[53] Whitby, G. S., Some New Reactions for the Detection of Sterols, *Biochem. J.*, 17: 5–12 (1923).

[54] Schoenheimer, R., Dam, H., and von Gottberg, K., The Absence of Allocholsterol in the Organism, *J. Biol. Chem.*, 110: 659–665 (1935).

[55] Bloor, W. R., The Determination of Cholesterol in Blood, *J. Biol. Chem.*, 24: 227–231 (1916).

[56] Girardin, R., and Spach, E., Sur le microdosage pondéral du cholestérol, *Bull. soc. chim. biol.*, 8: 813–815 (1926).

[57] Ransom, F., Saponin und sein Gegengift, *Deut. med. Wochschr.*, 27: 194–196 (1901); *cf.* also Hausmann, W., Über die Entgiftung des Saponins durch Cholsterin, *Beitr. chem. Physiol.*, 6: 567–580 (1905); and Abderhalden, E., and Le Count, E. R., Die Beziehungen zwischen Cholsterin, Lecithin und Cobragift, Tetanustoxin, Saponin und Solanum, *Z. exptl. Path. Therap.*, 2: 199–215 (1906).

as it does with digitonin. Lipolytic enzymes are inhibited or at least their reaction is retarded by the presence of cholesterol. The mechanism involved is unknown.

In 1872 Schulze described a compound obtained from wool fat which he designated as isocholesterol and assigned to it the formula $C_{26}H_{44}O$. This name has been carried in the literature as a sterol characteristic of wool fat. However, in 1930, a reinvestigation [58] showed that "isocholesterol" was a mixture of two compounds "lanosterin," $C_{30}H_{50}O$, containing two double bonds, and "agnosterin," $C_{30}H_{48}O$, containing three double bonds. A further investigation [59] has shown that lanosterin is not a true sterol but rather belongs to the polyterpene group since it does not contain the five-membered ring characteristic of the sterols and on selenium reduction gives 1-2-8-trimethylphenanthrene and not Diels hydrocarbon. Accordingly, the name isocholesterol should be removed from the sterol group.

In 1934, Bergmann [60] isolated a new sterol from the nonsaponifiable matter of oysters, *Ostrea virginica*. The same compound was isolated from the clam, *Venus mercenaria*, and was named ostreasterol. It appears to be the only sterol present in the oyster and replaces cholesterol completely in this organism and perhaps in all members of the *Lamellibranchiata*. It has the formula $C_{29}H_{48}O$, possesses two double bonds, and is isomeric with the plant sterol, stigmasterol, since on catalytic reduction it yields the same saturated alcohol, sitostanol, as do sitosterol and stigmasterol. Bergmann points out that the above facts are very significant inasmuch as this represents the first case in which a sterol derived from an animal source has been shown to be convertible into a typical sterol of plant origin, and he raises the question whether there is a sharp line of demarcation between the plant and animal sterols. He notes that, so far as the sterols are concerned, the oysters and other molluscs more nearly resemble the vegetable than the animal kingdom. Likewise this represents the first record of an animal organism devoid of cholesterol.

A new animal sterol, microcionasterol, has been isolated [61] from the sponge, *Microciona prolifera*. It has the formula $C_{27}H_{46}O$ and contains one double bond. Its structural relationships to the other sterols have not been ascertained. Another animal sterol, actinia-

[58] Windaus, A., and Tschesche, R., Über das sogenannte "Isocholsterin" des Wollfettes, *Z. physiol. Chem.*, 190: 51–61 (1930).

[59] Schulze, H., Über die Abgrenzung der Sterine gegenüber anderen Alkoholen der Polyterpen-Reihe und über den Bau des Lanosterins und Onocerins, *Z. physiol. Chem.*, 238: 35–53 (1936).

[60] Bergmann, W., Contributions to the Study of Marine Products. II. The Sterols of Mollusks, *J. Biol. Chem.*, 104: 316–328 (1934); and III. The Chemistry of Ostreasterol, *ibid.*, 104: 553–557 (1934).

[61] Bergmann, W., and Johnson, T. B., Beiträge zur Chemie der Meerestiere. I. Untersuchungen an dem Schwamm Microciona prolifera, *Z. physiol. Chem.*, 222: 220–226 (1933).

sterol, $C_{27}H_{44}O$, and containing two double bonds, apparently occurs [62] in the sea anemone, *Anemonia sulcata.*

A number of sterols have been isolated from plant materials. In the older literature these have been described under the general head of phytosterols. At least four of the plant sterols have been sufficiently characterized so that they are known to be individual substances. A number of the others which have been described may be individual substances or mixtures of one or more of the well-known plant sterols. Here again in the plant sterols the structural relationships have been clarified within the last few years. In all the plant sterols the double bond between carbon-5 and carbon-6 of the cholesterol nucleus is present.

In sitosterol, $C_{29}H_{50}O$, which occurs in the fats of the higher plants, the only difference from the structural formula of cholesterol is that a C_2H_5 group replaces a hydrogen on carbon-24 of the cholesterol nucleus. Stigmasterol, $C_{29}H_{48}O$, which occurs in the calabar bean, the soybean, and apparently in a considerable variety of the higher plants, differs from sitosterol in possessing an additional double bond situated between carbon-22 and carbon-23 on the aliphatic side chain.

Fucosterol, $C_{29}H_{48}O$, is isomeric with stigmasterol; it has an ethyl group on carbon-24 and two double bonds, one of which is between carbon-5 and carbon-6. It differs from stigmasterol in having the other bond somewhere in the nucleus and not on the aliphatic side chain. It seems probable that this double bond may be between carbon-7 and carbon-8 as in ergosterol. Fucosterol appears to be a sterol characteristic of the algae.[63] It has been isolated from *Fucus vesiculosus* and *Pelvetia canaliculata* of the brown algae, and *Nitella opaca* of the fresh-water green algae.

Ergosterol, $C_{28}H_{44}O$, was first isolated from the ergot fungus. Later it was found to be present in yeast, and yeast is the usual source from which it is derived commercially. It has three double bonds. It has the characteristic double bond between carbon-5 and carbon-6 as in cholesterol, sitosterol, stigmasterol, and fucosterol. It also has a double bond between carbon-22 and carbon-23 that is present in stigmasterol, and in addition has a third double bond between carbon-7 and carbon-8. It further differs from stigmasterol in that it has a methyl group rather than an ethyl group on carbon-24.

Two additional plant sterols are accepted by Fieser [64] as probably individual entities. These are cinchol, $C_{29}H_{50}O$, possessing one double

[62] Klenk, E., and Diebold, W., Actiniasterin, ein neues Sterin des Tierreiches, *Z. physiol. Chem.*, 236: 141–144 (1935).

[63] Heilbron, I., Phipers, R. F., and Wright, H. R., The Chemistry of the Algae. I. The Algal Sterol Fucosterol, *J. Chem. Soc.*, 1572–1576 (1934).

[64] Fieser, L. F., Chemistry of Natural Products Related to Phenanthrene, Second Edition, Reinhold Publishing Corporation, New York (1937).

bond, and present in cinchona bark, and zymosterol, $C_{27}H_{44}O$, possessing two double bonds and present in yeast. Neither one of these sterols has as yet been sufficiently characterized to indicate the relationships of their structures to the other members of the group.

THE STEROLS AND VITAMIN D.—A great stimulus to research in the field of the sterols followed the discovery of Rosenheim and Webster,[65] Steenbock and Black,[66] and Hess, Weinstock, and Helman,[67] who noted independently and at approximately the same time that apparently pure samples of cholesterol, when irradiated with ultraviolet light, acquired the properties of replacing the antirachitic vitamin, vitamin D, in the diet of an animal. Following these discoveries many workers irradiated oils and fats from various plant and animal sources, irradiated various foodstuffs and various sterols, and found rather generally that the sterol fraction acquired antirachitic properties by irradiation. As the study progressed, however, it was noted that all samples of sterols did not acquire the antirachitic property to the same degree, and a number of workers found almost simultaneously that cholesterol which had been purified by chemical methods had lost the property of becoming antirachitic when irradiated.

Prior to this time, Hess, Weinstock, and Sherman [68] had noted that irradiated cholesterol lost its antirachitic properties when it was recrystallized, and that the apparently pure cholesterol, which could be obtained from the recrystallization of irradiated cholesterol, could not again be activated by irradiation. They also noted [69] that after irradiation all the sterol could not be precipitated by digitonin. At about the same time Rosenheim and Webster [70] observed that only the sterols obtained directly from plant or animal tissues, such as cholesterol, sitosterol, and ergosterol, could be activated by irradiation. The group of "excretory" sterols, including coprosterol, were not activated by irradiation, indicating that the presence of an unsaturated linkage was necessary in order that the substance should acquire antirachitic properties.

[65] Rosenheim, O., and Webster, T. A., Rickets and Cholesterol, *Lancet*, 208: 1025–1026 (1925).

[66] Steenbock, H., and Black, A., Fat-Soluble Vitamins. XXIII. The Induction of Growth-Promoting and Calcifying Properties in Fats and Their Unsaponifiable Constituents by Exposure to Light, *J. Biol. Chem.*, 64: 263–298 (1925).

[67] Hess, A. F., Weinstock, M., and Helman, F. D., The Antirachitic Value of Irradiated Phytosterol and Cholesterol. I., *J. Biol. Chem.*, 63: 305–308 (1925).

[68] Hess, A. F., Weinstock, M., and Sherman, E., The Antirachitic Value of Irradiated Cholesterol and Phytosterol. IV. Factors Influencing Its Biological Activity, *J. Biol. Chem.*, 66: 145–160 (1925).

[69] Hess, A. F., Weinstock, M., and Sherman, E., The Antirachitic Value of Irradiated Cholesterol and Phytosterol. V. Chemical and Biological Changes, *J. Biol. Chem.*, 67: 413–423 (1926).

[70] Rosenheim, O., and Webster, T. A., The Anti-Rachitic Properties of Irradiated Sterols, *Biochem. J.*, 20: 537–544 (1926).

Hess and Anderson,[71] in a study of Anderson's α-, β-, and γ-sitosterols, found that the α-sitosterol became strongly antirachitic following irradiation, whereas the β- and γ-sitosterols acquired no antirachitic properties. At the time their paper was published the suggestion had been made that ergosterol was the precursor of vitamin D. They note that their β- and γ-sitosterols had been purified by chemical process (bromination) and suggest that possibly this process had destroyed any precursor of the antirachitic factor which may originally have been present in these fractions as an impurity. Rosenheim and Webster further noted that, when cholesterol was purified by chemical means and had lost the property of becoming antirachitic by irradiation, it likewise no longer possessed a characteristic absorption spectrum band in the ultraviolet region[72, 73] which is characteristic for cholesterol purified only by recrystallization. It appeared, therefore, as if some impurity were present in cholesterol purified by physical means and that the presence of this impurity accounted for the characteristic ultraviolet absorption band and for the acquiring of antirachitic properties by the cholesterol preparation.

Accordingly Rosenheim, Webster, Hess, and Windaus began an intensive investigation as to the nature of the impurity which might be present in the various sterol preparations and which might act as the provitamin of vitamin D. These workers were attacking the problem in different laboratories, but through a friendly agreement, they were all kept informed of the progress of the work in their several institutions. Almost simultaneously Windaus and Hess,[74] and Rosenheim and Webster[75] announced the finding that neither cholesterol which contains one double bond, sitosterol containing one double bond, nor stigmasterol containing two double bonds acquired antirachitic properties on irradiation following their purification by chemical methods. They did observe, however, that ergosterol, which contains three double bonds and which Rosenheim and Webster[76] had previously found to be after irradiation "highly protective even in doses of 1 mg.,"

[71] Hess, A. F., and Anderson, R. J., The Antirachitic Value of Irradiated Cholesterol and Phytosterol. VIII. The Activation of Sterol Fractions by Ultra-Violet Irradiation, *J. Biol. Chem.*, 74: 651–657 (1927).

[72] Schlutz, F. W., and Ziegler, M. R., Some Spectroscopic Observations on Cod Liver Oil, *J. Biol. Chem.*, 69: 415–419 (1926).

[73] Morton, R. A., Heilbron, I. M., and Kamm, E. D., The Absorption Spectrum of Ergosterol in Relation to the Photosynthetic Formation of Vitamin D, *J. Chem. Soc.*, 2000–2005 (1927).

[74] Windaus, A., and Hess, A., Sterine und antirachitisches Vitamin, *Nachr. Ges. Wiss. Göttingen, Math.-physik. Klasse*, 1926: 175–184.

[75] Rosenheim, O., and Webster, T. A., On the Nature of the Parent Substance of Vitamin D, *Lancet*, 212: 306–307 (1927).

[76] Rosenheim, O., and Webster, T. A., The Anti-Rachitic Properties of Irradiated Sterols, *Biochem. J.*, 20: 537–544 (1926); and The Parent Substance of Vitamin D, *ibid.*, 21: 389–397 (1927).

was apparently present in small amounts in all the sterol preparations which could be activated by irradiation, and they announced that the provitamin of vitamin D is ergosterol.

It seemed at this point as though one of the major problems associated with the chemistry of vitamin D had been solved. However, in later work it was found that the product (calciferol) derived from irradiated ergosterol and thought for a time to be the naturally occurring vitamin D, did not possess the same antirachitic properties as are evidenced by the vitamin D of cod-liver oil. The problems involved were attacked by a great many workers, and Bills [77] has been particularly active in this work. The historical phases will be reserved for treatment when we consider the chemistry and biological activity of vitamin D. Suffice it to say at this point that the antirachitic potency of the naturally occurring vitamin D is probably not due to the presence of a single compound, but there are apparently a number of antirachitic substances more or less closely related. When ergosterol is irradiated with ultraviolet light, ring II of the sterol is opened [78] between carbon-9 and carbon-10. Carbon-9 has two hydrogens attached to it, and a new double bond is formed [79] between carbon-10 and carbon-19, the methyl group at carbon-19 being converted to a methylene group. Somewhat later Windaus, Lettré, and Schenck [80] prepared 7-dehydrocholesterol by forming a double bond between carbon-7 and carbon-8 of the cholesterol molecule, and found that this compound not only gave the same ultraviolet absorption spectrum as ergosterol but that it, like ergosterol, could be converted by ultraviolet light into an antirachitic substance which was more potent than calciferol when tested on chicks. The present belief is that any sterol possessing double bonds between carbons-5-6 and 7-8 can acquire antirachitic properties when acted upon by ultraviolet light, and this probably explains the apparent multiplicity of antirachitic vitamins which occur in natural liver oils.

THE ISOLATION OF STEROLS.—The sterols are contained in the unsaponifiable matter of fats and oils, and are usually isolated by saponifying the fats, drying the soaps which are formed, and extracting these dry soaps with dry ether. The sterols are extracted by the ether, whereas the soaps are insoluble. The sterols are not water soluble, but in the saponified fat are dispersed as an emulsion in the presence of the soap solution. Cottonseed oil and corn oil yield about 1 per

[77] Bills, C. E., Physiology of the Sterols, Including Vitamin D, *Physiol. Rev.*, 15: 1–97 (1935).

[78] Lettré, H., Über die Ultraviolettbestrahlung des Ergosterins, *Z. angew. Chem.*, 47: 736 (1934).

[79] Kuhn, R., and Möller, E. F., Katalytische Mikro-Hydrierung organischer Verbindungen, *Z. angew. Chem.*, 47: 145–149 (1934).

[80] Windaus, A., Lettré, H., and Schenck, F., Über das 7-Dehydro-cholesterin, *Ann.*, 520: 98–106 (1935).

cent of unsaponifiable matter. Olive oil yields somewhat less than 1 per cent, and wheat oil about 2.5 per cent. Fish and liver oils may contain as much as 10 to 20 per cent of unsaponifiable matter. In such oils, however, other substances, largely hydrocarbons, are present, so that the unsaponifiable matter does not represent the sterol content.

THE BIOLOGICAL SYNTHESIS OF STEROLS.—It has been known for a long time that the vegetable sterols can be synthesized by both the lower and the higher plants. In the higher plants, such synthesis apparently can take place at any stage in the development of the plant. Until recently it has been assumed that the higher animals in some way transformed the plant sterols into cholesterol. However, in 1925, Channon [81] and Randles and Knudson [82] independently demonstrated that cholesterol could be synthesized in the animal body. Young rats were placed on a diet free from cholesterol and from any other sterols, at least so far as the absence of the Liebermann-Burchard reaction was concerned. These animals reproduced, and their young were continued on a sterol-free diet. The analysis of adult rats which had been born and raised on a sterol-free diet showed that relatively large quantities of cholesterol were present in the animal at birth and that larger amounts accumulated during the growth process.

Additional evidence that cholesterol is synthesized in the animal organism has been adduced by Schoenheimer,[83] who has demonstrated (1) that cholesterol is readily absorbed from the intestinal tract; (2) that coprosterol and dihydrocholesterol are not absorbed from the intestinal tract; (3) that the plant sterols, sitosterol, stigmasterol, and even ergosterol, are not absorbed from the intestinal tract; (4) that the source of dihydrocholesterol in the feces is through an excretion from the body into the intestinal tract and that its origin is not due to bacterial action; (5) that coprosterol is not a normal constituent of the body sterols, nor is it a normal constituent of plant sterols but is apparently formed in the intestinal tract by bacterial action, although no bacterium has as yet been isolated which can convert cholesterol into coprosterol *in vitro*; (6) that the body synthesizes its cholesterol and probably its own sterol which acts as a precursor for vitamin D. Schoenheimer suggests that the mechanism may be a dehydrogenation of cholesterol to produce the more unsaturated ergosterol and a simultaneous hydrogenation of cholesterol to produce dihydrocholesterol.

[81] Channon, H. J., Cholesterol Synthesis in the Animal Body, *Biochem. J.*, 19: 424–432 (1925).

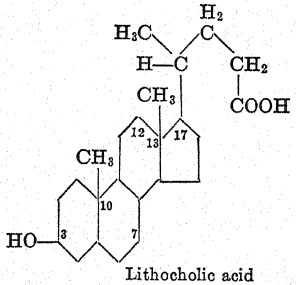
[82] Randles, F. S., and Knudson, A., Studies on Cholesterol. I. Synthesis of Cholesterol in the Animal Body, *J. Biol. Chem.*, 66: 459–466 (1925).

[83] Schoenheimer, R., New Contributions in Sterol Metabolism, *Science*, 74: 579–584 (1931).

The fact that cholesterol is readily absorbed from the intestinal tract, whereas the other sterols are not absorbed, is a striking example of the specificity of biological functions and of the importance of chemical configuration in the regulation of such biological functions. The mechanism involved in this selective absorption still remains to be elucidated. Obviously it is not one of simple diffusion.

THE BILE SALTS

The bile of the mammals is a golden brown to greenish fluid with an alkaline reaction, secreted by the liver and stored in the gall bladder. From the gall bladder it passes in small amounts into the intestines, where its chief function apparently is to emulsify fats and oils and to promote their hydrolysis and the absorption of the fatty acids by the intestinal mucosa. Bile contains inorganic salts, bile pigments, and salts of the bile acids. Although the bile acids are not found in fats and oils and are characteristic of bile, nevertheless their structure is so similar to that of the sterols that it seems justifiable to consider them in connection with the chemistry of the sterols. All the common bile salts have the sterol nucleus with a hydroxyl group on carbon-3 as in cholesterol, but the aliphatic side chain on carbon-17 is shortened, the break occurring between carbon-24 and carbon-25 with the conversion of carbon-24 into a carboxyl group, as shown in the formula for lithocholic acid. Desoxycholic acid has an additional hydroxyl group on carbon-12. Chenodesoxycholic acid is isomeric with desoxycholic acid but has its two hydroxyl groups on carbon-3 and carbon-7.



Lithocholic acid

Cholic acid has three hydroxyl groups situated at carbon-3, carbon-7, and carbon-12. These are the four common bile acids. In bile they occur as the sodium salts and are conjugated with either glycine or taurine.

The sodium salts of the bile acids, and particularly sodium desoxycholate, possess the remarkable property of forming association complexes [84] with many water-insoluble materials which thereby become

[84] Wieland, H., and Sorge, H., Untersuchungen über die Gallensäuren. II. Zur Kenntnis der Choleinsäure, *Z. physiol. Chem.*, 97: 1–27 (1916).

water-soluble. Thus, hydrocarbons, such as naphthalene and xylol are readily "dissolved" by aqueous solutions of the bile salts. Camphor, the natural fats and oils, cholesterol, etc., are likewise rendered water-soluble and brought into a form in which they are rather readily diffusible through membranes. The association complexes which are formed by the bile salts and compounds of the type that we have been discussing are relatively stable. For example, the stearic acid-desoxycholic acid complex dissolves without change in alkali, and the stearic acid is so firmly bound that it can be split off completely only by drastic oxidation or dehydration of the bile acid.

THE ANALYSIS OF FATS AND OILS

Various special technics have been devised for the study of the fats and oils. Some of the methods are essentially empirical, but have nevertheless been retained by the industry.

We can give only a brief outline of some of the more important of the technics. For the detailed methods, some of the analytical handbooks should be consulted.[85]

In research studies special technics are necessary for the separation of the glycerides and for the elucidation of the structure of the individual glycerides. Böeseken[86] has presented a most excellent résumé of separation technics which have been used during the past fifteen years to elucidate the structure of glyceride molecules. His paper is much more extensive than the title would indicate and covers a general survey of (1) physical and chemical methods for determining the characteristics and composition of the glycerides, (2) the chemical composition of some of the more important fats and oils, and, (3) some of the non-glyceride constituents which occur in fats and oils.

THE EXTRACTION OF THE FATS OR OILS FROM THE PLANT OR ANIMAL TISSUES.—In order completely to extract the fats or oils from the tissues, it is necessary that the tissues be dried, and *the drying must take place without oxidation of the fat or oil.* Various drying methods have been proposed, *e.g.*, tissues have been dried by heat in an air

[85] Association of Official Agricultural Chemists, Official and Tentative Methods of Analysis, Fourth Edition, Washington, D. C. (1935); Holland, E. B., Reed, J. C., and Buckley, J. P., Improved Methods for Fat Analysis, Bull. 166, Massachusetts Agr. Exp. Sta. (1915); Tentative Standard Methods for the Sampling and Analysis of Commercial Fats and Oils, *J. Ind. Eng. Chem.*, 10: 315–320 (1918); Abderhalden, E., *Hanb. biol. Arbeitsmeth.*, Abt. I., Chemische Methoden, Teil 6, Lipoide, Säuren, Cyclosen (1925); and Jamieson, G. S., Vegetable Fats and Oils, Chemical Catalog Company, New York (1932), *cf.* Chapter VI, Methods, pp. 321–407.

[86] Böeseken, J., Researches on Vegetable Oils in the Chemical Laboratory of the Technical University of Delft, *Comptes-rendus*, Vol. II, Ve Congrès International Technique et Chimique des Industries Agricoles, Scheveningue (1937), pp. 348–366.

oven, or by heat *in vacuo*, or by heat in a neutral gas, such as nitrogen. Drying by heat in air is not to be recommended, and whenever possible the drying should be done *in vacuo* or in the presence of a neutral gas. In some instances, it has been found desirable to dry material by adding absolute alcohol to abstract the water and, at a low temperature, distilling off the dilute alcohol so formed. In other instances, anhydrous calcium sulfate (plaster of Paris) or anhydrous sodium sulfate is used to combine with the water, the water being bound in the form of water of crystallization. In still other instances, notably in the case of meat samples, the material has been frozen, and then the frozen material dried *in vacuo* over sulfuric acid, the desiccator being kept at a temperature below zero. Meat can be dried in this way with practically no oxidation of the fats, the meat sample retaining almost its original size and shape.

Following the drying procedure, the fat or oil is extracted by some appropriate solvent. In general, dry neutral ether is chosen as the solvent, although chloroform or carbon tetrachloride has been used by some investigators. In the event that the material contains a high percentage of protein, it is sometimes necessary to follow the original ether extraction by extraction with absolute alcohol, and this in turn by a second ether extraction. This is notably the case where fat is adsorbed upon protein surfaces or where fats or fat-like compounds are combined with proteins to form the so-called lecithoproteins or lipoproteins. Following extraction, the solvent is removed by evaporation at low temperature, leaving behind the sample of fat or oil for examination.

Physical Properties Which May Be Determined on the Fat or Oil Samples.—A number of physical properties have been used to characterize fats and oils. These include:

1. The specific gravity, which may be determined by means of the hydrometer or preferably a pycnometer or a Westphal balance.

2. The melting point, which is obtained by suspending a disc of the solid fat approximately midway in an aqueous solution which has been adjusted so as to have approximately the same specific gravity as the fat. This solution is then slowly heated, and the point is noted at which the disc of fat "rounds up" into a globule. This so-called melting point is more or less indefinite, because a fat is composed of a number of substances having different melting points. Accordingly it is seldom worth while to undertake the rather tedious determination of the so-called melting point.

3. The titre test, *i.e.*, the temperature at which the fatty acids, which have been liberated from the fat after the fat has been saponified by alkali, resolidify.

4. The refractive index, taken either with an Abbé butyro refrac-

tometer or a dipping refractometer. The refractive index is a very important and valuable index of the composition and purity of a fat or oil.

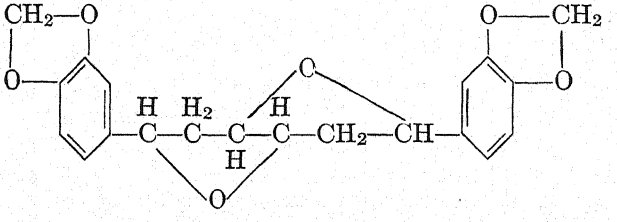
5. The optical rotation may be taken; sometimes it gives important data.

6. The viscosity, which is again an important criterion, is very largely used in the industry. The Saybolt or the MacMichael viscosimeters are more commonly employed.

7. The specific heat: In the event that the fat or oil is to be used for purposes of lubrication, it is sometimes desirable to obtain the value for the specific heat.[87]

COLOR REACTIONS.—Numerous color reactions have been described, by which an oil from one plant or animal source may be distinguished from similar oils from other plant or animal sources. Gill,[88] however, points out that color reactions can be regarded only as *circumstantial evidence*, inasmuch as they depend upon impurities carried along into the fat or oil from its natural source. The glycerides of one plant or animal species are, in general, not different from those of other species of plants or animals. Accordingly there is no color test which will distinguish a pure fat or oil from other fats and oils. Lewkowitsch [89] likewise has a very poor opinion of color tests as indicating the original source of an oil.

Sesame oil gives a very characteristic color in the presence of a trace of furfural and concentrated hydrochloric acid. The color has been shown [90] to be due to the presence of a compound, sesamoline, $C_{20}H_{18}O_6$, which on hydrolysis yields sesamol, the 3-4-methylene ether of 1-3-4-tri-hydroxybenzene. Sesamoline has the structure:



where the sesamol appears to be united in the molecule in an unusual glycosidal-like linkage.

[87] Wesson, D., and Gaylord, H. P., Specific Heat of Fats and Oils, *Cotton Oil Press*, 2: 40–41 (1918).

[88] Gill, A. H., Color Tests for Oils—Palm Oil, *J. Ind. Eng. Chem.*, 9: 136–139 (1917).

[89] Lewkowitsch, J., Chemical Technology and Analysis of Oils, Fats, and Waxes, Fourth Edition, 3 Vols., Macmillan and Company, London (1909).

[90] Adriani, W., Über Sesamin und Sesamolin, *Z. Untersuch. Lebensm.*, 56: 187–194 (1928); *cf.* also Böeseken, J., and Cohen, W. D., Das Sesamin, *Biochem. Z.*, 201: 454–463 (1928).

QUALITATIVE TESTS FOR PARTICULAR CLASSES OF FATS.—1. As already noted, oleic acid is transformed into its isomer, elaidic acid, by treatment with nitric oxide. Accordingly the *elaidin test* is a qualitative test for the presence of oleins. An oil containing a large percentage of olein becomes a solid fat on treatment with nitric oxide.

2. The linoleic and linolenic acids of the drying oils, when treated with bromine, form characteristic tetrabromides and hexabromides (*vide infra*). This reaction has given rise to the so-called *hexabromide test* for drying oils. This point will be referred to later.

CHEMICAL METHODS.—A number of chemical methods have been proposed for the characterization of fats and oils.

1. *The Acid Number.*—The acid number may be defined as the milligrams of potassium hydroxide necessary to neutralize the free fatty acids present in 1 gram of a fat, oil, or wax.

2. *The Iodine Absorption Number.*—This may be defined as the number of centigrams of iodine taken up by 1 gram of fat. In other words, it may be expressed as the percentage of iodine taken up by 1 gram of fat.

3. *The Saponification Number or the Koettstorfer Number.*—This is the milligrams of potassium hydroxide necessary to saponify 1 gram of a fat or oil.

4. *The Total Fatty Acids.*—Five grams of the fat or oil are saponified, and the fatty acids precipitated as a lead soap, using lead acetate solution. The lead soaps are washed and then decomposed by shaking with 1 : 5 hydrochloric acid, liberating the free fatty acids. These are dissolved in anhydrous ether. The ethereal solution is dried, and an aliquot of the solution evaporated, and the residue weighed.

5. *The Reichert-Meissl Number.*—This is the number of cubic centimeters of 0.1 *N* potassium hydroxide required to neutralize the volatile acids from 5 grams of a saponified fat or oil.

6. *The Hehner Number.*—This is the weight of the non-volatile (insoluble) fatty acids yielded by 5 grams of a saponified fat or oil.

7. *The Polenske Number.*—This is the number of cubic centimeters of 0.1 *N* potassium hydroxide required to neutralize the non-volatile fatty acids obtained from 5 grams of a saponified fat or oil. The residue remaining in the flask from the Reichert-Meissl determination is usually used to determine the Polenske number.

8. *The Acetyl Number.*—This is the milligrams of potassium hydroxide necessary to combine with the acetic acid liberated by the saponification of 1 gram of acetylated fat or oil.

9. *Thiocyanogen Number.*—Kaufmann [91] observed that whereas

[91] Kaufmann, H. P., Die Rhodanometrie von Fetten und Fettgemischen, *Z. Untersuch. Lebens*, 51: 15-27 (1926); *cf.* also Zeleny, L., and Bailey, C. H., Thiocyanogen Number and Its Application to Studies on Lard, *Ind. Eng. Chem.*, 24: 109-110 (1932).

bromine and iodine add to *both* of the double bonds of linoleic acid, as well as to the single bond of oleic acid, *thiocyanogen*, $(SCN)_2$, *while it adds to the double bond of oleic acid, adds to only one of the double bonds of linoleic acid.* It therefore is possible to calculate the amount of linoleic acid in a mixture by determining the ratio between the iodine number or the bromine number and the thiocyanogen number. The thiocyanogen reagent is prepared by treating lead thiocyanate in glacial acetic acid with the calculated amount of bromine. Lead bromide is formed, and the thiocyanogen remains dissolved in the glacial acetic acid. The solution used should be about 0.05 molar. The paper by Zeleny and Bailey may be consulted for details of the analysis and for methods of calculation. The use of the thiocyanogen number has demonstrated that the more reactive double bond of linoleic acid is preferentially hydrogenated before there is any appreciable hydrogenation of the double bond of oleic acid.

THE SEPARATION, IDENTIFICATION, AND ESTIMATION OF THE INDIVIDUAL CONSTITUENTS OF AN OIL, FAT, OR WAX.—Such a study constitutes in reality a research problem.[92] In general, one may expect to go through somewhat the following procedure, although no definite technic can be given which should be followed irrespective of the nature of the material under investigation.

1. The fat or oil is saponified, and the saponification is followed by drying the soaps and removing the non-saponifiable residue by extracting the dry soaps with anhydrous ether. The fatty acids are then set free from the soaps and separated by distillation into the volatile fatty acids and the non-volatile fatty acids. Dry and preserve the solid non-volatile acids in petroleum ether, and use as noted under 4.

2. The mean molecular weight of the volatile and the non-volatile fatty acids is determined by titrating an aliquot with standard alkali.

3. The volatile fatty acids are separated into fractions as follows: The soaps are prepared, and these are treated with 20 per cent sulfuric acid. (*a*) The caproic and the higher acids separate as an oil which is removed. (*b*) The remaining solution is saturated with sodium chloride, butyric acid separating as an oil. (*c*) The residual salt solution is extracted with ether to remove any of the lower fatty acids which may be present. The three fractions, (*a*), (*b*), and (*c*), of the volatile fatty acids which were obtained are then converted into silver, calcium, or barium salts. These salts are analyzed, and, from the percentage of silver, calcium, or barium which they contain, the probable composition of the fractions is determined.

4. The non-volatile fatty acids may be separated by any one of

[92] Baughman, W. F., and Jamieson, G. S., The Composition of Hubbard Squash Seed Oil, *J. Am. Chem. Soc.*, 42: 152–157 (1920): *cf.* also Jamieson, G. S., and Baughman, W. F., Okra Seed Oil, *ibid.*, 42: 166–170 (1920); and Ball, C. D., Jr., A Study of Wheat Oil, *Cereal Chem.*, 3: 19–39 (1926).

three methods, (*a*) the lead salt-ether method or (*b*) the fractional precipitation of the barium or magnesium salts, or (*c*) the fractional distillation of the esters of the acids *in vacuo*.

The lead salts of the *saturated* fatty acids are insoluble in ether, whereas the lead salts of the *unsaturated* fatty acids are soluble in ether. Accordingly the lead salt-ether method is, in general, used to separate the higher saturated fatty acids from the unsaturated fatty acids, although both the lead salt-ether method and the fractional precipitation or fractional distillation may be necessary.[93] The hydroxamic acids[94] formed when hydroxylamine is added to a mixture of fatty acids ($R—COOH + NH_2OH = R—CO—NH—OH + H_2O$) may sometimes be used to advantage. The sodium salts of stearohydroxamic acid and palmitohydroxamic acid are insoluble in alcohol, whereas the corresponding sodium salt of oleohydroxamic acid is freely soluble. The differences are so marked that Lewis states a quantitative separation to be possible. The sodium salts of the saturated fatty acids are insoluble when the chain contains twelve or more carbon atoms, and soluble for acids containing eight or less carbon atoms. Lauric acid (C_{12}) forms a somewhat soluble sodium hydroxamic salt.

5. The liquid, unsaturated fatty acids may be separated by means of their brom derivatives or by means of their oxidation products.

(*a*) *Separation by Means of Brom Derivaties.*—The unsaturated fatty acids are brominated in glacial acetic acid. Organic solvents are then used to separate the bromine compounds into groups showing different solubilities. The oleic group (one double bond) of fatty acids yields dibrom derivatives which are soluble in petroleum ether. The linoleic group (two double bonds) yields tetrabrom derivatives, insoluble in petroleum ether but soluble in ethyl ether. The linolenic group (three double bonds) yields hexabrom derivatives, insoluble in petroleum ether, insoluble in ethyl ether, but soluble in hot benzene.

(*b*) *Separation by Means of Oxidation Products.*—The separation of the liquid, unsaturated fatty acids through their oxidation products is carried out by oxidizing the fatty acids to their corresponding hydroxy acids. The linolenic series yields hexahydroxystearic acids which are soluble in cold water. The linoleic series yields tetrahydroxystearic acids, soluble only in large quantities of boiling water. The oleic series yields dihydroxystearic acids, insoluble in hot water,

[93] Holland, E. B., Reed, J. C., and Buckley, J. P., Determination of Stearic Acid in Butter Fat, *J. Agr. Research*, 6: 101–113 (1916); and Holland, E. B., and Buckley, J. P., Determination of Fatty Acids in Butter Fat: I. *ibid.*, 12: 719–732 (1918).

[94] Lewis, A. H., The Separation of Fatty Acids, *Biochem. J.*, 20: 1356–1363 (1926).

but soluble in ethyl ether. The influence of polar hydroxyl groups is very marked in the above solubility series.

6. The solid, saturated fatty acids are separated by the fractional distillation of their methyl and ethyl esters. In many instances, the esters have to be refractionated a number of times, providing any considerable number of the higher saturated fatty acids are present. The various fractions are then converted into their silver salts. These are crystallized and analyzed for silver as a test for the purity of the various fractions.

7. The glycerol content of the liquor from which the fatty acids were removed is determined by some appropriate method.

8. The non-saponifiable residue is further studied for the presence of the higher alcohols, including the sterols. The methods are largely those of fractional crystallization or fractional distillation, combined with the formation of chemical derivatives which may serve for identification purposes.

CHAPTER XXXIV

THE COMPOUND LIPIDS

MacLean and MacLean [1] and Thierfelder and Klenk [2] have presented an excellent discussion of this group of compounds. The compound lipids are substances of a fat-like nature, yielding on hydrolysis fatty acids or derivatives of fatty acids and containing in the molecule either nitrogen or phosphorus and nitrogen.

Our knowledge of the organic phosphorus compounds occurring in both plants and animals is still very limited and incomplete. At least six groups of organic phosphorus compounds occur in nature.

1. *Phytin* or *phytic acid*, in which phosphoric acid is combined with inositol as inositol phosphoric acid, phytin being the calcium-magnesium salt of inositol phosphoric acid. Inositol is hexahydroxyhexahydrobenzene, and occurs very widely distributed throughout the plant kingdom, notably in the pericarp of seeds. The phosphoric acid combined with the inositol probably functions as a reserve phosphorus supply. The inositol itself has become of added interest following the observation of Eastcott [3] that inositol is bios I, a substance necessary for the normal reproduction of the yeast cell.[4] A compound analogous to inositol, but a derivative of hexahydrotoluene instead of hexahydrobenzene, is mytilit, isolated [5] from the mussel, *Mytilus edulis*. Its physiological importance is unknown. Sequoyite, a monomethyl ester of *i*-inositol, occurs in the heartwood of the redwood.[6]

2. *The hexose phosphates*, according to Harden,[7] and Harden and

[1] MacLean, H., and MacLean, I. S., Lecithin and Allied Substances, Second Edition, The Lipins, Longmans, Green and Company (1927).

[2] Thierfelder, H., and Klenk, E., Die Chemie der Cerebroside und Phosphatide, Julius Springer, Berlin (1930).

[3] Eastcott, E. V., Wildiers' Bios. The Isolation and Identification of "Bios I," *J. Phys. Chem.*, 32: 1094–1111 (1928).

[4] Wildiers, E., Nouvelle substance indispensable au développement de la levûre, *La Cellule*, 18: 313–333 (1901); *cf.* also, Miller, W. Lash, Bios. *J. Chem. Education*, 7: 257–267 (1930).

[5] Ackermann, D., Über den Mytilit, eine natürlich vorkommende Cyclose, *Ber.*, 54: 1938–1943 (1921).

[6] Sherrard, E. C., and Kurth, E. F., Sequoyite, A Cyclose from Redwood (*Sequoia sempervirens*), *J. Am. Chem. Soc.*, 51: 3139–3141 (1929).

[7] Harden, A., Alcoholic Fermentation, Fourth Edition, Longmans, Green and Company, London (1932).

Young,[8] are intermediary products in the alcoholic fermentation by yeast.

3. *Nucleic acids* have already been discussed. Here phosphoric acid is combined with carbohydrates and purine and pyrimidine bases.

4. *The phosphoproteins* have likewise already been discussed. The best-known examples are casein and vitellin. The phosphorus is present in the protein molecule as phosphoric acid, esterified on the hydroxyl group of the hydroxyamino acids. The phosphoproteins, so far as we are aware, occur only in the animal kingdom.

5. *Creatine-phosphoric acid* (phosphagen) was discussed when we considered the mechanism of muscle contraction. The corresponding *argininephosphoric acid* which replaces phosphagen in crustacean muscle likewise has been noted.

6. There remain *the phospholipids* (the phosphatides of Thudichum). In contrast to the groups which we have noted, the phospholipids are soluble in fat solvents. The best-known example of this group is probably "lecithin," although the "lecithins" which have been most widely studied probably do not represent a pure compound. In lecithins and cephalins, the phosphoric acid occurs esterified on one of the hydroxyl groups of glycerol. In sphingomyelins, the phosphoric acid is esterified on the hydroxyl group of a nitrogenous base, sphingosine.

THE PHOSPHOLIPIDS.—This group may be further subdivided into (1) the *monoaminomonophospholipids*, having a ratio of 1 atom of nitrogen to 1 atom of phosphorus, of which lecithin and cephalin are the known examples; and (2) the *diaminomonophospholipids*, having the ratio of 2 nitrogen atoms to 1 atom of phosphorus, and being represented by sphingomyelin. In all probability these three compounds are the only compounds occurring in the phospholipid group, and although claims have been made in the literature for other compounds, nevertheless none of these claims have been substantiated.

The work in the field of the compound lipids dates from the publication by Thudichum, in 1874, of a paper entitled, "Researches on the Chemical Constitution of the Brain."[9] Thudichum continued to publish in this difficult field of chemical research for a number of years. Of recent years many workers have contributed to this field, chief among whom should be noted P. A. Levene, H. MacLean, O. Rosenheim, E. Klenk, and H. Thierfelder.

Probably no field of biological chemistry offers more difficulties than the study of the separation and identification of the chemical

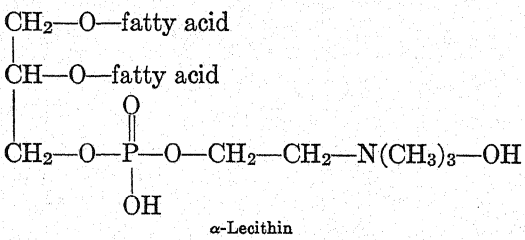
[8] Harden, A., and Young, W. J., The Alcoholic Ferment of Yeast-Juice, *Proc. Roy. Soc.* (*London*), B., 77: 405–420 (1906).

[9] Thudichum, J. L. W., Researches on the Chemical Constitution of the Brain, Report of the Medical Officer of the Privy Council and Local Government Board, New Series, 3: 113–247 (1874).

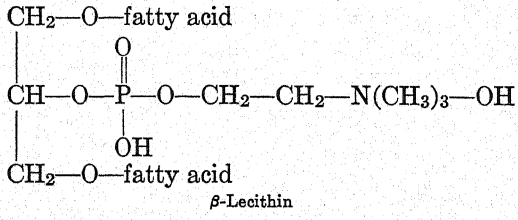
structure of the compound lipids which comprise such a large fraction of the nervous tissue.

The phospholipids upon hydrolysis yield phosphoric acid, various fatty acids, and basic substances, such as choline (trimethyl-β-ethyl alcohol ammonium hydroxide) or β-aminoethyl alcohol. The phospholipids are soluble in ordinary fat solvents but are *insoluble in and precipitated by acetone.* They form crystallizable addition products with platinic chloride and with cadmium chloride. The preparations may be hydrophilic, even hygroscopic, and imbibe water to form colloidal sols.

The Lecithins.—On hydrolysis, lecithins yield 1 molecule of glycerolphosphoric acid, 2 molecules of fatty acids, and 1 molecule of choline. The choline is attached to the glycerolphosphoric acid by esterification of the hydroxyl group of the oxyethyl radical with the phosphoric acid. The fatty acids are esterified on the two remaining hydroxyl groups of glycerol. It is evident that, if only one of the hydroxyl groups of glycerol is esterified with phosphoric acid, two isomeric glycerolphosphoric acids are possible. In one of these, phosphoric acid would be esterified in the α-position, and such a compound should be optically active. In the other, phosphoric acid would be esterified in the β-position and the glycerolphosphoric acid would not be optically active. Glycerolphosphoric acid as it has been obtained from a number of lecithins shows optical activity, but there is abundant evidence that both α- and β-lecithins occur in nature.

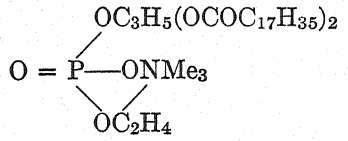


α-Lecithin

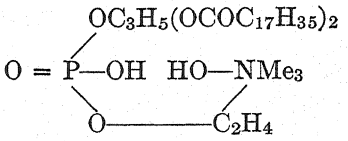


β-Lecithin

Lecithins can exist both in the hydrated form and in the anhydride form where water has been lost between the residual acid group of the phosphoric acid and the hydroxyl group of the substituted ammonium hydroxide.



Anhydride form of lecithin



Hydrated form of lecithin

In the analyses of various lecithins which have been reported by different investigators, we find records of the presence of stearic, palmitic, oleic, linoleic, and arachidonic acids. Since there are only two molecules of fatty acids in a lecithin, it is evident that a considerable number of isomeric "lecithins" are possible, particularly in view of the fact that both α- and β-forms offer additional opportunities for isomerism. It thus appears almost certain that the term lecithin does not mean a specific chemical compound but rather that it is the generic name of a group of compounds possessing similarity in structure but likewise possessing specific differences in the nature of the fatty acids esterified in the molecule. Thudichum stated that all true lecithins contain 1 molecule of an unsaturated fatty acid and 1 molecule of a saturated fatty acid. Levene's studies point in the same direction, and most workers agree that, in general, this appears to be so, although recently some evidence has been brought forward for a distearyllecithin and dioleyllecithin. There seems to be no *a priori* reason why the fatty acids associated with the lecithin molecule should of necessity be different, and perhaps we are dealing here with the same general rule that by and large simple glycerides rarely occur in nature. The difficulty of definitely settling even this problem lies in the great ease with which lecithins hydrolyze and the still greater ease with which they oxidize. Lecithins, when pure, have a translucent, paraffin-like, colorless appearance, but they quickly turn yellow on exposure to air and even in a very few minutes may become an opaque brown. Therefore if a "pure" lecithin preparation is tested, the product must be prepared without exposure to air at any stage in the process. Lecithins are constituents of all cells so that they apparently have a very important function in life processes. It has been suggested that one of their functions is to assist in regulating the permeability of the cell membrane. The lecithins are excellent emulsifying agents and probably play a role in the maintenance of protoplasmic structure. Furthermore, they readily form coordination complexes with proteins, and in the form of lecithoproteins must play important physiological roles.

Grün and Limpächer [10] claim to have synthesized a lecithin by first treating distearin (or other diglycerides) with one molecule of phosphorus pentoxide and then treating the product with two mole-

[10] Grün, A., and Limpächer, R., Synthese der Lecithine, I, *Ber.*, 59: 1350–1360 (1926); II, *ibid.*, 60: 147–150 (1927).

cules of choline bicarbonate or some similar salt of choline. The lecithin was isolated by neutralizing its benzene solution with alcoholic alkali, thus separating cholinephosphoric acid, and then precipitating the lecithin with acetone, thus separating it from residual distearin. The product precipitated with acetone analyzed for lecithin and had the characteristic physical and chemical properties of lecithin.

Lecithins are readily hydrolyzed by lipase. Contardi and Ercoli [11] point out that there are really four distinct lecithinases corresponding to the four types of ester linkages in lecithin.

Lecithinase-A liberates only one molecule of fatty acids. This enzyme occurs in cobra venom and rattlesnake venom.[12] When one fatty acid is split off from a lecithin, the residual product is known as lysolecithin. Lysolecithin is powerfully hemolytic, and there is evidence that the hemolytic action of cobra venom lies in the ability of the venom to form lysolecithin within the organism.

Lecithinase-B, which ocurs in rice hulls, splits both fatty acid molecules from the lecithin molecule. Lecithinase-C can apparently hydrolyze off only the choline radical, and lecithinase-D is a true glycerolphosphatase and breaks the bond between the phosphoric acid and the glycerol molecule.

The Cephalins (*Kephalins*).—These are compounds usually associated with the lecithins and distinguishable from the lecithins by the fact that they are *insoluble in alcohol* and when hydrolyzed yield aminoethyl alcohol instead of choline. Presumably they possess the same structural configuration as the lecithins but contain a different nitrogenous base. The separation of the lecithins and the cephalins is extremely difficult, for although pure lecithin is soluble in alcohol and pure cephalin is insoluble in alcohol, a mixture of the two does not sharply separate in absolute alcohol. Bull and Frampton [13] found that, in the presence of lecithins, the cephalins were fairly soluble in alcohol, and Bull [14] suggests that the soluble mixture possibly represents a coacervate system.

In the cephalins we again are probably dealing with a group of compounds, as in the lecithins. Various workers have reported the

[11] Contardi, A., and Ercoli, A., Über die enzymatische Spaltung der Lecithine und Lysocithine, *Biochem. Z.*, 261: 275–302 (1933).

[12] King, E. J., and Dolan, M., The Enzymic Hydrolysis of Phosphatides. II. Lysolecithin, *Biochem. J.*, 27: 403–409 (1933); *cf.* also King, E. J., The Enzymic Hydrolysis of Lecithin, *ibid.*, 25: 799–811 (1931); and The Enzymic Hydrolysis of Phosphatides. III. The Hydrolysis of Natural and Synthetic Phosphatides, *ibid.*, 28: 476–481 (1934).

[13] Bull, H. B., and Frampton, V. L., Physico-Chemical Studies on Lecithin, *J. Am. Chem. Soc.*, 58: 594–596 (1936).

[14] Bull, H. B., The Biochemistry of the Lipids, John Wiley & Sons, New York (1937).

same fatty acids to occur in the cephalins that have been found in the lecithins.

Grün and Limpächer [15] claim to have synthesized cephalin using a procedure analogous to that which they used with lecithin. The compound which they synthesized, however, contained two molecules of stearic acid, whereas the products isolated by Levene [16] contained one molecule of stearic acid with the other molecule either oleic or arachidonic acid. Magistris [17] prepared lysocephalin and found that it possessed very little hemolytic power. He suggests that in all probability lysocephalin does not possess hemolytic power and that the hemolysis noted in his preparations was due to the presence of small amounts of lysolecithin.

The Sphingomyelins.—These phospholipids occur in large quantities in the brain and nerve tissue, and in smaller quantities can be isolated from most of the organs of the body. They yield as decomposition products [18] phosphoric acid, lignoceric acid, cerebronic acid, stearic acid, and two nitrogen bases, choline and sphingosine. Sphingosine appears to be an unsaturated diatomic amino alcohol having the structure $C_{13}H_{27}$—CH=CH—CHOH—CHOH—CH_2NH_2.

Some of the problems involved in the constitution of the sphingomyelin molecule have not been solved. Levene proposes that the fatty acid is conjugated with the amino group and that the phosphoric acid is esterified on the α-hydroxy group and that the choline in turn is esterified on the phosphoric acid. Since sphingomyelins contain only one fatty acid radical, and, since at least three fatty acids have been isolated from sphingomyelins, it is evident that there are at least three sphingomyelins: a stearylsphingomyelin, a lignocerylsphingomyelin, and a nervonylsphingomyelin. It is interesting to note that Thudichum suggested that there were probably several sphingomyelins.

Nervonic acid, $C_{24}H_{46}O_2$, is an unsaturated fatty acid having one double bond, $CH_3(CH_2)_7CH{=}CH{-}(CH_2)_{13}COOH$. The formula thus indicates that it is the singly unsaturated acid corresponding to the saturated C_{24} lignoceric acid and bears the same relationship to lignoceric acid that oleic acid bears to stearic acid. There is an accumulation of sphingomyelins in the brain, liver, and spleen of in-

[15] Grün, A., and Limpächer, R., Synthese der Kephaline, *Ber.*, 60: 151–156 (1927).

[16] Levene, P. A., and Rolf, I. P., Unsaturated Fatty Acids of Brain Cephalins, *J. Biol. Chem.*, 54: 91–98 (1922).

[17] Magistris, H., Über die hämolytische Wirkung einiger Abbauprodukte des Lecithins, der Lecithide und Phosphatide, *Biochem. Z.*, 210: 85–119 (1929).

[18] Levene, P. A., Spyingomyelin, I., *J. Biol. Chem.*, 15: 153–154 (1913); II, *ibid.*, 18: 453–462 (1914); and III, *ibid.*, 24: 69–89 (1916); *cf.* also Levene, P. A., and Taylor, F. A., The Synthesis of α-Hydroxyisopentacosanic Acid and Its Bearing on the Structure of Cerebronic Acid, *ibid.*, 52: 227–240 (1922).

fants suffering from Niemann-Pick's disease. This disease of infancy is marked by an enlarged spleen and liver, the spleen showing lipid deposits, by anemia, and by a leukocytosis with a marked increase in lymphocytes. It is usually associated with idiocy and blindness where there is no demonstrable lesion in the structure of the eye. The cerebrosides appear to be absent in this disease and to be replaced by sphingomyelins.

Sphingomyelins are white, crystalline, non-hygroscopic substances relatively stable to light and air. They can be distinguished from the lecithins and cephalins by being insoluble in ether and also by the absence of glycerol among the hydrolytic products. They become hydrated and emulsified in water.

SULFUR-CONTAINING PHOSPHOLIPIDS.—Thudichum isolated a sulfur-containing phospholipid from brain tissue which he called cerebrosulfatide. This was further studied by Koch,[19] who observed that the compound contained equal molecular amounts of sulfuric acid and phosphoric acid. Fränkel and Karpfen[20] have recently made a further study of this compound. They give the empirical formula as $C_{101}H_{152}N_3PSO_{26}$, having a ratio of S : P : N of 1 : 1 : 3, all the nitrogen being in the form of amino groups. They identified, as hydrolytic products, phosphoric acid, sulfuric acid, glycerol, and aminoethyl alcohol. All the nitrogen was present in the form of the aminoethyl alcohol, choline and sphingosine being completely absent. The only fatty acid which appeared to be present was a hitherto unknown hydroxy acid, α-hydroxy-*n*-decanic acid, $C_{10}H_{20}O_3$.

Fränkel and Gilbert[21] had previously isolated from human brain substance another phospholipid containing sulfur, the compound having the formula $C_{93}H_{191}N_3SPO_{18}$. The hydrolytic products of this compound were phosphoric acid, sulfuric acid, glycerol, aminoethyl alcohol, and cerebronic acid. Just how these various decomposition products are linked in these lipids remains to be determined. These sulfur-containing compounds of Fränkel have not been generally accepted by other workers. MacLean refers to them as "alleged lipids," and it may be that they represent a coacervate of a true phospholipid with other sulfur-containing compounds.

THE CEREBROSIDES.—The cerebrosides are variously referred to in the literature as cerebrosides (Thudichum), galactolipins (Leathes and MacLean), and galactosides (Rosenheim). They are characterized by containing one molecule of sphingosine, one molecule of *d*-galactose,

[19] Koch, W., Zur Kenntnis der Schwefelverbindungen des Nervensystems. II. Über ein Sulfatid aus Nervensubstanz, *Z. physiol. Chem.*, 70: 94–97 (1910).

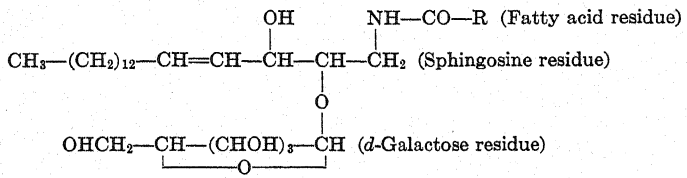
[20] Fränkel, S., and Karpfen, O., Über die Hypohirnsäure, *Biochem. Z.*, 157: 414–424 (1925).

[21] Fränkel, S., and Gilbert, O., Über die Darstellung von Phosphorsulfatiden aus Gehirn, *Biochem. Z.*, 124: 206–215 (1921).

and one molecule of a C_{24} (or C_{25}) fatty acid. The fatty acid is attached to the amino group of sphingosine through an amide ("peptide") linkage, since there is no free amino group present in the cerebroside and the *d*-galactose can be split off leaving the sphingosine-fatty acid compound.

If the fatty acid is split off, there remains a non-reducing compound, *psychosine*, consisting of one molecule of sphingosine and one molecule of *d*-galactose with the amino group free. The *d*-galactose is combined with one of the hydroxyl groups of sphingosine through a glycosidal linkage. The *d*-galactose is the normal pyranose form.[22]

Three cerebrosides are known with certainty, and Klenk has produced evidence for a fourth. All have the same fundamental structure, the only difference being in the nature of the fatty acid residue.



In this formula of the cerebrosides there are still several points which are uncertain. It is not known with certainty which hydroxyl group is involved in the linkage between the sphingosine and *d*-galactose, nor is it known whether the amino group is on carbon-1, carbon-2, or carbon-3 of the sphingosine molecule. These three carbons carry the amino group and the two hydroxyl groups. It appears as if there is a chance here for isomeric forms of the various cerebrosides.

The three cerebrosides which are generally recognized are *phrenosin* (Thudichum, 1879) or *cerebron* (Thierfelder, 1905), *kerasin* (Thudichum, 1874; Rosenheim,[23] 1916), and *nervon* (Klenk, 1925). The fatty acid of phrenosin is cerebronic acid (phrenosinic acid), which according to Levene[24] is a hydroxy acid having the formula $C_{25}H_{50}O_3$, and according to Klenk[25] is the α-hydroxy-*n*-C_{24} acid. The acid of kerasin is lignoceric acid, apparently the *n*-C_{24} acid. The

[22] Pryde, J., and Humphreys, R. W., The Nature of the Sugar Residue of the Cerebrosides of Ox-Brain, *Biochem. J.*, 20: 825–828 (1926).

[23] Rosenheim, O., The Galactosides of the Brain. IV. The Constitution of Phrenosin and Kerasin, *Biochem. J.*, 10: 142–159 (1916).

[24] Levene, P. A., and West, C. J., On Cerebronic Acid, I, *J. Biol. Chem.*, 14: 257–265 (1913); IV, *ibid.*, 18: 477–480 (1914); and Levene, P. A., and Taylor, F. A., VI, *ibid.*, 80: 227–230 (1928).

[25] Klenk, E., Über eine Säure $C_{24}H_{46}O_3$ aus Cerebrosiden des Gehirns, *Z. physiol. Chem.*, 157: 291–298 (1926); Über die Nervonsäure, *ibid.*, 157: 283–290 (1926); Über die Cerebroside des Gehirns, *ibid.*, 166: 268–286 (1927); Über die Nervonsäure, *ibid.*, 166: 287–293 (1927); Über die Oxysäuren der Cerebroside des Gehirns, *ibid.*, 174: 214–232 (1928); Über die Cerebronsäure, *ibid.*, 179: 312–319 (1928).

acid of nervon is nervonic acid,[26] a singly unsaturated n-C_{24} acid with the double bond between carbons 15-16. In his studies of the cerebrosides Klenk isolated a fourth acid which he calls oxynervonic acid.[27] According to Klenk the four acids occurring in the cerebrosides differ only slightly in characteristic structure:

Lignoceric acid, $CH_3(CH_2)_{22}COOH$
Cerebronic acid, $CH_3(CH_2)_{21}CHOH—COOH$
Nervonic acid, $CH_3(CH_2)_7CH=CH—(CH_2)_{13}COOH$
Oxynervonic acid, $CH_3(CH_2)_7CH=CH(CH_2)_{12}CHOH—COOH$

We have already noted that Levene does not agree with Klenk's formula for cerebronic acid and believes that it is a C_{25} acid, perhaps with a branch chain. A recent contribution to this controversy is the study [28] of the fatty acids of pure phrenosin and kerasin, using X-ray technic and melting-point studies. From this study it is concluded that cerebronic acid is a mixture of C_{22}, C_{24}, and C_{26} hydroxy-n-acids and that lignoceric acid likewise is a mixture of the C_{22}, C_{24}, and C_{26}-n-acids. A synthetic mixture of the C_{22}, C_{24}, and C_{26}-n-acids in the molar ratios of 1:8:1 gave a melting point and an X-ray pattern identical with the "lignoceric" acid isolated from kerasin. If these observations prove to be correct, it is apparent that there are several phrenosins, kerasins, and nervons.

As might be anticipated, the various cerebrosides show very similar chemical properties. When dry, they are white and more or less wax-like and separate from alcohol in microscopic particles which give the superficial appearance of crystal balls. They apparently, however, are not truly crystalline, although claims have been made for obtaining phrenosin in true crystal form. Under proper experimental conditions they readily form "liquid crystals" which probably accounts for the great divergence of data in the literature regarding their "melting point." The cerebrosides are not limited to the brain or nervous tissue but have been reported to occur in the spleen, kidneys, liver, egg yolk, blood corpuscles, lungs, adrenals, the retina of the eye, fish sperm, and even in plant sources such as fungi, seeds, and the heart wood of oak. In Gaucher's disease the amount of cerebrosides in the liver and spleen is greatly increased. Aside from the isolation of cerebrosides from the spleen and liver in Gaucher's disease the general occurrence of these compounds outside of nerve tissue, especially from plant sources, should be looked upon with suspicion.

[26] Klenk, E., Über ein neues Cerebrosid des Gehirns, *Z. physiol. Chem.*, 145: 244–260 (1925).

[27] Klenk, E., *Z. physiol. Chem.*, 174: 214–232 (1928).

[28] Chibnall, A. C., Piper, S. H., and Williams, E. F., The Fatty Acids of Phrenosin and Kerasin, *Biochem. J.*, 30: 100–114 (1936).

No cerebroside approaching in purity preparations made from brain and nerve tissue has as yet been isolated from plant sources, and it is possible that some of the old literature dealt with impure preparations of phospholipids contaminated with carbohydrate materials. Page[29] has brought together our knowledge of the brain lipids, and his book together with that of Thierfelder and Klenk can be consulted for further data.

THE PLANT LIPIDS.—Our present knowledge of the plant lipids is very limited. We do not know with certainty whether or not plant and animal lipids are identical in chemical constitution. The chief studies of plant lipids have centered around the phospholipids, inasmuch as these can be more readily followed in the course of fractionation by studying the percentage of the phosphorus. Dried seeds of legumes may contain as much as 1.5 per cent of phospholipids; the cereal grains usually contain approximately 0.5 per cent.

Carbohydrates appear to be more generally present in the plant lipids than in animal lipids. Plant lecithin entirely free from carbohydrate has not as yet been obtained, although the hydrolysis products of plant lecithin, *i.e.*, glycerol, phosphoric acid, choline, and aminoethyl alcohol, are the same as are yielded by the animal lecithins. Levene and Rolf[30] have investigated the plant phospholipids of the soybean. The lecithin fraction yielded on hydrolysis stearic and palmitic acids, oleic acid, and acids belonging to the linoleic and linolenic series. No unsaturated acid containing more than eighteen carbon atoms could be isolated. They likewise found, in addition to lecithin, a compound having the properties of cephalin, which appears to be similar to, if not identical with, the cephalin isolated from animal sources. The lecithin likewise was found to contain a carbohydrate which was identified as a pentose, on the basis of color reactions with orcinol. Aminoethyl alcohol was readily isolated as the aurichloride.

In regard to the biological functions of the lipids in general, we only know that the phospholipids are essential constituents of all cells, which indicates that they must be essential for the life process.[31] The fact that they are all good emulsifying agents suggests the possible function of maintaining the proper colloidality of protoplasm. They are all more or less toxic if given in larger doses than can be readily assimilated by the tissues.

[29] Page, I. H., Chemistry of the Brain, C. C. Thomas Company, Springfield, Illinois (1937).

[30] Levene, P. A., and Rolf, I. P., Plant Phosphatides. I. Lecithin and Cephalin of the Soy Bean, II. Lecithin, Cephalin, and So Called Cuorin of the Soy Bean, *J. Biol. Chem.*, 62: 759–766 (1925); 68: 285–293 (1926).

[31] Levene, P. A., The Chemical Individuality of Tissue Elements and Its Biological Significance, *J. Am. Chem. Soc.*, 39: 828–837 (1917).

CHAPTER XXXV

ESSENTIAL OILS

The essential oils may be defined as "those compounds in plants which are volatile with steam and usually separate as an oily layer in the distillate." They are present to a small extent in most plants and may be present to a very considerable extent in certain families or groups of plants. Some essential oils are of interest only from the scientific standpoint, involving the identification or the preparation of rare organic compounds. Others such as oils of wintergreen, clove, cinnamon, bergamot, attar of roses, lemon and orange oils, camphor, cedar oil, pine oil, eucalyptus oil, turpentine, etc., are of very considerable commercial importance.

In certain plants essential oils may occur in all the tissues. This is notably true of the conifers. In the rose they occur in appreciable amounts only in the petals, in cinnamon only in the bark and leaves, in the orange in the petals of the flowers and the skin of the fruit, in the nutmeg chiefly in the fruit, and in the camphor tree both in the leaves and throughout the entire woody tissue.

The function of essential oils in the plant kingdom is unknown. Here again, various suggestions have been made, as in the case of the tannins, that they may be waste products, or protection against injury or the invasion of fungi, and that they may provide an odor which will attract insects so as to favor pollination. Very closely allied species may differ greatly in their content of essential oils and in the chemical constituents present in the essential oils.

Miller[1] investigated the essential oils of three species of *Pycnanthemum, P. tullia, P. incanum,* and *P. lanceolatum.* Miller notes that it is very difficult to sharply separate these three species on morphological grounds. He found, however, that the compounds present in the essential oils were widely different. Thus, the essential oil of *P. tullia* consisted of approximately 50 per cent of cineol; that from *P. incanum* consisted of approximately 90 per cent of pulegone, whereas the main fraction of the oil from *P. lanceolatum* consisted of cavacrol, only approximately 5 per cent of pulegone being present. Miller accord-

[1] Miller, E. R., A Chemical Investigation of the Volatile Oils of Some Species of the Genus *Pycnanthemum* Michx., Ph.D. thesis on file in the Library, University of Minnesota, and privately printed (1918).

ingly suggests in his discussion that a study of the essential oils might well be undertaken in certain families as at least an assistance in determining botanical classifications.

McNair [2] studied the chemical composition of 398 essential oils from 87 plant families with respect to physical properties and the chemical compounds which were present in the essential oils. He concludes that the nature of the essential oils is intimately related to the position of the plant in the phylogenetic scale, that terpenes and compounds of the fatty series predominate in the volatile oils produced by the plants lowest in the evolutionary scale, and that the plants highest in the evolutionary scale contain more of the volatile oils with aromatic, sulfur, and nitrogenous compounds. He points out also that there is almost a straight-line relationship between the refractive index of the volatile oil and the position of the plant on the evolutionary scale, those plants high in the evolutionary scale having volatile oils with a low refractive index. A similar relationship was found to hold for specific gravity; the higher the evolutionary scale of the plant, the greater was the specific gravity of the essential oil. His studies also brought out the fact that there is a climatic influence on the nature of the compounds which are produced. The essential oils of the tropics have lower specific gravities and higher refractive indices than these of temperate climates. He concludes by insisting that the chemical composition of the plant, including particularly the nature of the alkaloids, glycerides, and essential oils, be taken into consideration in taxonomic revision of the various plant groups, since these three groups of chemical compounds appear to form a scale against which the relative degree of evolution of the various groups can be charted.

The field of the chemistry of the essential oils presents many exceedingly difficult problems. Some oils consist almost wholly of a single compound. Other oils are mixtures [3] containing a dozen or more compounds of the most diverse types.

The physical methods for identification of essential oils are somewhat similar to those already noted for the usual fats and oils, such as (1) optical rotation, (2) specific gravity, (3) index of refraction, and (4) boiling-point range, *i.e.*, the temperature range over which an oil can be completely distilled.

[2] McNair, J. B., Some Properties of Plant Substances in Relation to Climate of Habitat—Volatile Oils, Saponins, Cyanogenetic Glucosides, and Carbohydrates, *Am. J. Bot.*, 19: 168–193 (1932); The Evolutionary Status of Plant Families in Relation to Some Chemical Properties, *ibid.*, 21: 427–452 (1934); and Angiosperm Phylogeny on a Chemical Basis, *Bull. Torrey Botan. Club.*, 62: 515–532 (1935).

[3] Power, F. B., The Distribution and Characters of Some of the Odorous Principles of Plants, *J. Ind. Eng. Chem.*, 11: 344–352 (1919).

The laboratory methods[4,5] of separating the compounds present in the mixture are in part:

1. Low temperatures, such as freezing point.—By this method it is sometimes possible to crystallize out certain of the constituents.

2. Fractional distillation with steam serves to separate an oil into the more volatile and the less volatile fractions.

3. Fractional distillation *in vacuo* of the dried oil again may serve to bring about a partial separation, as may

4. Crystallization from poor solvents of certain oils and fractions of certain oils.

5. The constituents of an essential oil possessing free acidic groups may be removed by shaking the oil with sodium carbonate solution and separating the aqueous solution of the sodium salts so formed from the main bulk of the oil.

6. Similarly, basic compounds may be removed by shaking the oil with a dilute solution of hydrochloric acid and removing the aqueous acid solution containing the bases.

7. Phenols may be removed by shaking the oil with a dilute solution of sodium or potassium hydroxide.

8. Aldehydes and ketones may be removed by shaking the oil with a saturated solution of sodium bisulfite, resulting in the formation of the crystalline bisulfite addition products which are non-oil-soluble.

9. By a determination of the acetyl value, the free hydroxyl groups which are present in the oil may be determined.

10. The acids which were separated by shaking with sodium hydroxide are liberated by the acidification of the solution and are converted into esters or into their silver or barium salts. The saponification number of the esters or the percentage of silver or barium in the salts provides a means of identifying the acids which are present.

11. In many instances, specific compounds which occur frequently in essential oils may be tested for by means of special color reactions.

Undoubtedly the average individual is chiefly interested in the essential oils as the basis of the perfume industry. The perfume industry is very highly developed in France. At Grasse, a city of approximately 20,000 inhabitants, the entire countryside is one flower garden. This city alone uses each year[6] 2,200 tons of orange blossoms, 1,650 tons of rose petals, 1,320 tons of jasmine petals, 440 tons of violets, 330 tons of tuberoses, 165 tons of carnations, 110 tons of cassie, 880 tons of mimosa branches, 66 tons of mignonette, and 55 tons of nar-

[4] Parry, E. J., The Chemistry of Essential Oils and Artificial Perfumes, Scott, Greenwood and Company, London (1899).

[5] Semmler, F. W., Die aetherischen Öle, 4 vols., Veit and Company, Leipzig (1906–1907).

[6] Bogert, M. T., The Flower and the Organic Chemist: Perfumes—Natural and Synthetic, *J. Ind. Eng. Chem.*, 14: 359–364 (1922).

cissus. One pound of orange blossom oil was obtained from 1,000 pounds of blossoms. One pound of attar of roses required 8 tons of petals.

Three methods of extraction are employed when the oils are to be made into perfumes.

1. *Extraction with Petroleum Ether.*—This gives a greater yield, but other substances are extracted along with the true essential oils, resulting in a lower grade of perfume.

2. *Maceration of the Material with Warm Oil or Fat.*—The material is ground in the presence of added oil or fats. The fat containing the essential oil is then expressed by means of a hydraulic press, and the essential oil recovered by extracting the fat with strong alcohol. The fat (lard) is largely insoluble in the alcohol and crystallizes out on cooling, leaving an alcoholic solution of the odoriferous principles. This is a better method than (1) but still yields an inferior grade of perfume for many flowers.

3. *Cold Absorption.*—Thin layers of cold fat (40 per cent beef, 60 per cent lard) are spread about ⅛ inch thick on both sides of glass plates, and a layer of petals about 2 inches deep is placed above these. The layers of fat and petals are racked up one on top of the other as high as the operator can reach. After 24 to 72 hours (depending upon the flower being used and the quality of perfume desired), the flowers are removed and new flowers added. Finally the layers of fat are extracted with *cold alcohol,* the fat which dissolves in the alcohol is frozen out and the alcoholic solution is concentrated or bottled directly. This process yields the finest perfumes.

The essential oils as used in industrial processes or in medical practice may be obtained by three general methods:

1. By some suitable solvent, such as petroleum ether, dichloroethylene, etc., the solvent being of such a nature that it can be readily and completely removed by evaporation at a low temperature.

2. In some instances, oils may be extracted by pressure, using ground material and a hydraulic press.

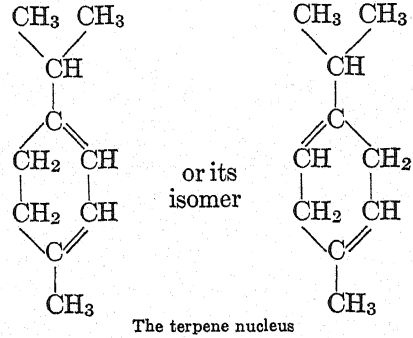
3. The more general method,[7] however, is that of steam distillation, steam from a boiler or superheated steam being passed through a mass of raw material which contains the essential oils. This steam passing through the material carries the oil over, the oil separating as a layer in the distillate.

As already noted, various types of chemical compounds, such as hydrocarbons, alcohols, ketones, aldehydes, acids, esters, organic sulfides, bases, etc., may occur in essential oils. Only a few typical exam-

[7] Sievers, A. F., Methods of Extracting Volatile Oils from Plant Material and the Production of Such Oils in the United States, *Tech. Bull.* 16, U. S. Dept. Agr. (1928).

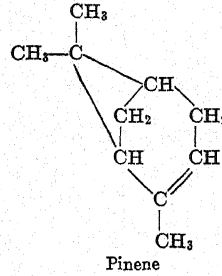
ples will be noted to illustrate the diversity of compounds which may be present.

I. *Terpenes.*—These are hydrocarbons of the general formula, $C_{10}H_{16}$, and in general are closely related to the parent hydrocarbon. The relationships of these configurations to the cyclic end-groups of the carotenoids should be noted.

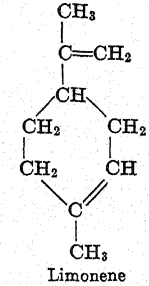


The terpene nucleus

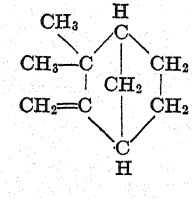
Certain of the typical terpenes are pinene (from conifers), limonene (from lemon-grass oil), and camphene (from the camphor tree).



Pinene

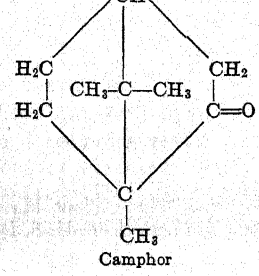


Limonene

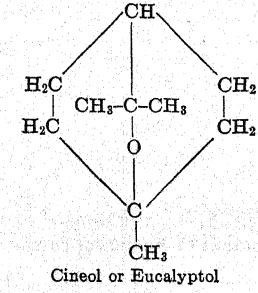


Camphene

II. *Alcohols and Ketones.*—These types of compounds are very abundant in essential oils. The camphor series includes camphor, borneol, pulegone, menthol, etc.

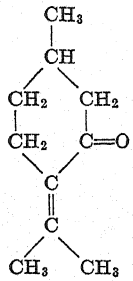


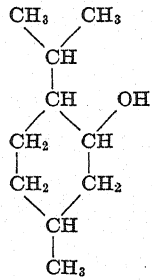
Camphor



Cineol or Eucalyptol

Camphor is obtained from the wood of the camphor tree. It has been synthesized, and the synthetic product is a serious competitor of the natural product. It would appear that the camphor industry faces at the present time the same problems that the natural indigo industry faced a few decades ago.

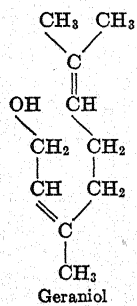

Pulegone

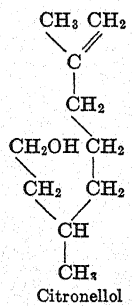

Menthol

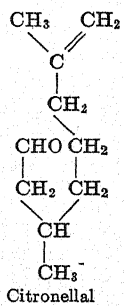
Borneol has the same structural groupings as camphor with the exception that the keto group is replaced by a secondary alcohol group.

Menthol occurs in oil of peppermint to the extent of 65 to 85 per cent, depending on the quality of the peppermint oil. The corresponding ketone, menthone, also occurs in peppermint oil. Cineol or eucalyptol is very widely distributed as a component of many of the essential oils and occurs in a very large amount in oil of eucalyptus.

III. *Geraniol and Citronellol Group* of alcohols, ketones, and aldehydes is another important group. Here we do not have a closed ring, but the formulas may be written so as to indicate their relationship to the closed ring structures, which have already been noted. It will be observed that here there is a marked similarity to the lycopin residue of the carotenoids.

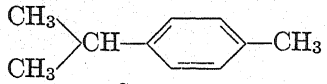

Geraniol


Citronellol

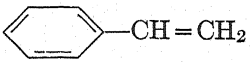

Citronellal

Geraniol occurs in oils of rose, geranium, sassifras, lavender, etc. Citronellal occurs in lemon-skin and in orange-skin oils, eucalyptus oil, lemon-grass oil, etc.

IV. *Benzene Hydrocarbons* (not terpenes) are of common occurrence, *e.g.*, cymene occurs in caraway oil, styrene in styrax balsam.

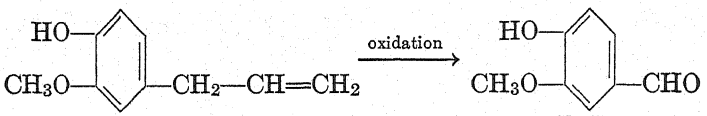


Cymene



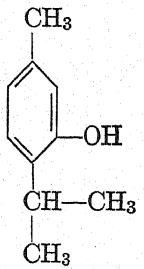
Styrene

V. *Phenols* are widely distributed in the essential oils. Thus, eugenol is the principal constituent of oil of cloves. On oxidation eugenol is converted to the corresponding aldehyde, vanillin, the flavoring principle of the vanilla bean.

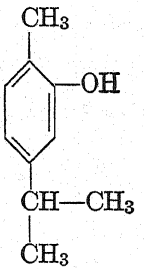


Eugenol Vanillin

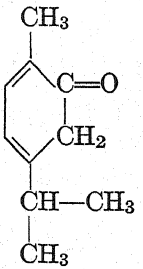
Thymol (isopropyl-*m*-cresol) occurs in a number of essential oils but particularly in the oil of thyme. It is a hydroxy derivative of cymene which is likewise found in oil of thyme and in eucalyptus oil. The corresponding *o*-cresol derivative is carvacrol, occurring in many essential oils, and the corresponding ketone of carvacrol is carvone.



Thymol



Carvacrol



Carvone

VI. *Acids and esters of acids* are of common occurrence. These include benzoic acid and its esters, salicylic acid and its esters. The esters of acetic, butyric, and valeric acids are of rather common occurrence. Methyl salicylate, the methyl ester of *o*-hydroxybenzoic acid, is practically the only constituent of pure oil of wintergreen and oil of birch. Ethyl acetate is the chief constituent of oil of banana. Amyl valerianate and ethyl anthranilate (the ethyl ester of *o*-aminobenzoic acid) are the chief constituents of oil of apples. Amyl butyrate occurs in oil of apricots. Ethyl salicylate, ethyl butyrate, and amyl acetate occur in the fruit of the strawberry.

VII. A number of the *aliphatic alcohols* are of common occurrence. Some of these are water-soluble and do not separate with the oil but

remain in the aqueous portion of the distillate, from which they must be removed by extraction with a suitable non-miscible solvent or by fractional steam distillation.

VIII. *Aliphatic aldehydes* in small amounts, such as traces of formaldehyde, acetaldehyde, etc., are of rather common occurrence.

IX. *Sulfides* occur in certain oils. Thus, allyl sulfide

$$CH_2{=}CH{-}CH_2{-}S{-}CH_2{-}CH{=}CH_2$$

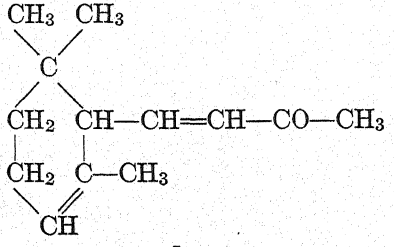
occurs in oil of garlic, and allyl thiocyanate

$$CH_2{=}CH{-}CH_2{-}N{=}C{=}S$$

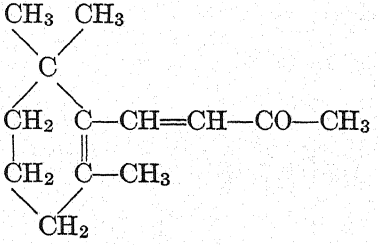
occurs in oil of mustard. Essential oils containing sulfur are especially abundant in the *Cruciferae*. In many instances the sulfur-containing radical is combined with sugars in the form of a glycoside. Thus, allyl thiocyanate occurs in mustard seed as a glycoside which must be hydrolyzed either by enzymatic action or by chemical means before the allyl thiocyanate can be distilled as an essential oil.

X. Small amounts of the *paraffin hydrocarbons*, C_nH_{2n+2}, are occasionally found in essential oils, but their occurrence is rare.

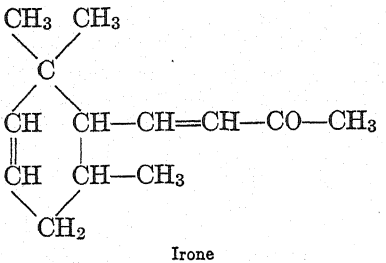
XI. Essential oils usually contain small amounts of *organic bases*. It is still a debatable question whether these are normal constituents of essential oils or have been derived from proteins, lipids, or other nitrogenous constituents of the plant by some decomposition process, either that of the natural autolysis of the plant material or a decomposition brought about by the methods used for preparing the essential oil. It seems probable that they are secondary decomposition products. The one possible exception is indole, which occurs in extremely small amounts in the oil of orange blossoms. Indole, when present in appreciable amount, has a very disagreeable and penetrating odor. The amount which is present in orange-blossom oil is, however, extremely small and in conjunction with the other constituents of the oil produces the characteristic orange-blossom perfume. The synthetic perfume failed to completely simulate the natural perfume until a trace of indole had been added.



α-Ionone



β-Ionone



Irone

Most of the perfumes which are sold to the consumer represent blends which are mixtures of natural essential oils, or blends of compounds which have been synthesized [8] in the organic laboratory. In some instances compounds have been synthesized which very closely resemble in their chemical structure the constituents occurring in the natural perfumes. Thus, α- and β-*ionone* were synthesized in 1893 in the attempt to synthesize *irone*, the compound responsible for the odor of orris root and probably oil of violets. A mixture of the synthetic α- and β-ionone, which differ from irone only in the position of the double bond,[9] produces a violet perfume so nearly like the natural product as to defy detection by all but the most expert of perfume connoisseurs. The ionones were regarded as wholly synthetic products until in 1929 β-ionone was shown [10] to be present in rather large amount in the essential oil of *Boronia megastigma*. Later Karrer showed the ionone residues to be the characteristic configurations in the carotenoids (*vide supra*), the ring of α-ionone occurring in α-carotene and that of β-ionone in β-carotene. Irone itself, though it has been known since 1893, was not synthesized [11] until 1935. While it is isomeric with the ionones, the position of the double bond confers on the molecule a much greater odor value than is possessed by the ionones.

[8] Bogert, M. T., Recent Progress in the Field of Synthetic Perfumes, *J. Chem. Education*, 8: 1311–1334 (1931).

[9] Hibbert, H., and Cannon, L. T., Condensation of Citral with Ketones and Synthesis of Some New Ionones, *J. Am. Chem. Soc.*, 46: 119–130 (1924).

[10] Sabetay, S., Sur la présence de la β-ionone dans un produit naturel, *Compt. rend.*, 189: 808–809 (1929).

[11] Verley, A., Sur la synthèse de l'irone de Tiemann, *Bull. soc. chim.*, [5] 2: 1205–1208 (1935).

CHAPTER XXXVI

THE HORMONES *

The word *hormone* was first applied to this group of *chemical messengers* by Bayliss and Starling [1] in 1902 at the suggestion of W. B. Hardy.[2] The word is derived from the Greek ορμαω, meaning, "I arouse to activity." The earlier term of "internal secretions" did not sufficiently indicate their special functions, and the terms "chemical messengers" or "excitants fonctionnels," while indicating physiological properties and special functions, did not seem satisfactory as a permanent nomenclature. The term hormone, as applied by Bayliss and Starling, was intended to designate *those chemical substances secreted by the endocrine glands which, when carried by the blood stream to another organ, profoundly influence the activity of that organ.*

In the highly complex mechanism representative of the bodily activities of the higher mammals, different organs and tissues have taken over very special activities and functions. In order that the entire organism may work in harmony special mechanisms have come into being. One of these mechanisms is the nervous system, which may be likened to an intercommunicating network of telegraph wires centering in the great central switchboards of the brain, spinal cord, and ganglia. In order, however, that life processes may proceed at a *uniform* rate rather than undergoing violent fluctuations as the environment changes, a second system of "chemical messengers" was necessitated whereby the increased activity of one organ would be reflected in an altered activity of another organ and thus, through a system of checks and balances, the complex mechanism would work as a unified whole. The hormones provide this "balance wheel." Their presence enables the organism to pass through periods of stress with all forces of the organism mobilized to meet the test and then, when the emergency has passed, to resume the normal coordination of bodily activities.

* Dr. Leroy S. Palmer assisted largely in the preparation of this chapter.

[1] Bayliss, W. M., and Starling, E. H., The Mechanism of Pancreatic Secretion, *J. Physiol.*, 28: 325–353 (1902).

[2] *Cf.* page 712, Bayliss, W. M., Principles of General Physiology, Fourth Edition, Longmans, Green and Company, London and New York (1924).

It should be emphasized that the endocrines are not independent of one another but are at least to a certain extent dependent on one another's secretions. Because of these interrelationships we can readily see how the entire function of the body may be stimulated, retarded, or perverted by the excess activity, lessened activity, or dysfunction of any one of the endocrine glands. That this is the case has been abundantly established by endocrinologists. Still more recently it has been discovered that certain of the endocrine glands secrete a multiplicity of hormones, and though this secretion probably arises in each instance from specific cells, nevertheless the unbalance of the organism with respect to a single hormone may account for what was formerly attributed to the dysfunction of the entire gland.

While the discoveries in the field of the hormones in the past decade have been all-important, one fact stands out as preeminent, *i.e.*, the apparent master-key control of all the other endocrine glands which resides in certain of the specific hormones elaborated by the anterior lobe of the pituitary. These master-key hormone-controlling hormones of the anterior pituitary appear to be proteins or protein-like in nature, and some have even speculated as to whether there may not possibly exist one grand-master hormone, likewise a protein, which is responsible for life itself.

There is no generally accepted classification of the hormones. For purposes of convenience, however, they may be divided into two groups: (*A*) those hormones having somewhat general physiological and metabolic functions, and (*B*) those having specific physiological and/or metabolic functions. There follows the listing of thirty-one individual hormones, for the existence of which there is a reasonable amount of data in the literature. Possibly some of these will be shown not to have hormonal characteristics; probably other as yet unknown hormones will be added to the list as the result of further research. In the brief space represented by this chapter it will be impracticable to discuss in detail all the chemical and physiological evidence for the hormones in this list. The interested reader will therefore have to turn to larger treatises[3] for many important details.

[3] Harrow, B., and Sherwin, C. P., The Chemistry of the Hormones, The Williams and Wilkins Company, Baltimore (1934); Cameron, A. T., Recent Advances in Endocrinology, Third Edition, P. Blakiston's Son and Company, Philadelphia (1936); Trendelenburg, Paul, Die Hormone, ihre Physiologie und Pharmakologie, Vols. I and II, Julius Springer, Berlin (1929–1934); *Annual Review of Biochemistry*, Vols. I-VI, Stanford University Press, Stanford University, California (1932–1937); Glandular Physiology and Therapy, A Symposium, American Medical Association, Chicago (1935); Internal Secretions, *Cold Spring Harbor Symposia on Quantitative Biology*, Vol. 5, The Biological Laboratory, Cold Spring Harbor, N. Y. (1937).

A. Hormones having somewhat general physiological and metabolic functions:

1. Thyroxine, thyroid
2. Epinephrine, adrenal medulla
3. Cortipressin, adrenal cortex
4. Thymus principle
5. Pineal principle
6. Pitocin (alpha-hypophamine or oxytocin), posterior pituitary
7. Pitressin (beta-hypophamine or vasopressin), posterior pituitary
8. Growth principle, anterior pituitary

B. Hormones having specific physiological and/or metabolic functions:

9. Parathormone, parathyroids
10. Insulin, pancreas
11. Cortin, adrenal cortex
12. Cortilactin, adrenal cortex
13. Prolactin, anterior pituitary
14. Thyrotropic principle, anterior pituitary
15. Parathyrotropic principle, anterior pituitary
16. Adrenotropic (interrenotropic) principle, anterior pituitary
17. Diabetogenic principle (blood-sugar raising), anterior pituitary
18. Diabetogenic principle (ketogenic), anterior pituitary
19. Pancreotropic principle, anterior pituitary
20. Gonadotropic principles (prolan A and prolan B), anterior pituitary
21. Estradiol, ovarian follicular fluid
22. Estrone, ovarian follicular fluid
23. Estriol, ovarian follicular fluid (from estradiol)
24. Equilin, from estrone
25. Equilenin, from estrone or equilin
26. Pregnandiol, corpus luteum of ovaries
27. Progesterone, corpus luteum (from pregnandiol)
28. Testosterone, testes
29. Androsterone, testes (from testosterone)
30. Emmenin(s), placenta (from estradiol)
31. Secretin, duodenal mucosa

THE HORMONES OF THE ADRENAL GLANDS.—The adrenal or suprarenal glands are small flattened bodies situated in the retroperitoneal tissue at the upper end of each kidney. Each gland consists of an internal medulla and an external cortex, which is encased in a capsule

or sheath of connective tissue. Both the medulla and cortex tissues secrete specific hormones.

The Hormones of the Medulla.—The cells of the medulla secrete a hormone known as epinephrine. The secreting cells are stained brownish-yellow by potassium chromate and have been designated chromaffine cells. Cells having similar staining reactions are found in ganglia and nerve cells of most invertebrates and contain a substance having physiological properties analogous to if not identical with those of epinephrine. Gaskell [4] suggests that these cells and their secretions in invertebrates are the progenitors of the epinephrine secretory chromaffine cells of the adrenal medulla and of the sympathetic nervous system of the higher vertebrates.

Epinephrine was the first hormone to be isolated and identified as a chemical entity. In 1898, Abel [5] isolated the compound as the benzoate and prepared a number of other derivatives. He also showed that the compound had great physiological activity. Abel gave to the compound the name *epinephrine*. At about the same time von Fürth [6] prepared potent extracts and isolated metallic derivatives of a compound which he called *suprarenine*. Somewhat later Takamine devised essentially the present commercial method for the preparation of epinephrine and called the substance *adrenalin* under which name it was marketed commercially. Epinephrine is β-hydroxy-β(3,4-dihydroxyphenyl)N-methylethylamine. Since it contains an asymmetric carbon atom, it is optically active. The *levo* form occurs in the adrenal gland, and is physiologically fifteen to twenty times as potent as the *dextro* form. The function of epinephrine is to maintain smooth muscle in a state of excitability so that it will readily react to nerve impulses transmitted by the sympathetic nerve fibers. Epinephrine contracts the capillary arteries, thereby exerting a marked effect upon the blood pressure. The entire contractile vascular system is regulated both by the secretion of epinephrine and by the sympathetic nervous system acting in conjunction. The hormone is exceedingly potent. The concentration of epinephrine normally present in the circulating blood has been estimated to be of the order of 1:1,000,000,000 to 1:2,000,000,000. Elliott,[7] Tscheboksaroff,[8] and Cannon,[9] and others

[4] Gaskell, J. F., Adrenalin in Annelids, *J. Gen. Physiol.*, 2: 73–85 (1919).

[5] Abel, J. J., Über den blutdruckerregenden Bestandtheil der Nebenniere, das Epinephrin, *Z. physiol. Chem.*, 28: 318–362 (1899).

[6] von Fürth, O., Zur Kenntniss der brenzkatechinähnlichen Substanz in den Nebennieren, *Z. physiol. Chem.*, 24: 142–158 (1898).

[7] Elliott, T. R., The Control of the Suprarenal Glands by the Splanchnic Nerves, *J. Physiol.*, 44: 374–409 (1912).

[8] Tscheboksaroff, M., Über sekretorische Nerven der Nebennieren, *Arch. ges. Physiol.*, 137: 59–122 (1911).

[9] Cannon, W. B., Bodily Changes in Pain, Hunger, Fear, and Rage, D. Appleton and Company, New York (1915) (Second Edition, 1929).

have supported the view that in periods of stress, such as fear, hunger, pain, rage, etc., the stimulation of the splanchnic nerve causes a discharge of larger amounts than usual of epinephrine into the blood stream, thus causing a rise in blood pressure and a general mobilization of the body forces which enables the organism to rise and meet the emergency. Furthermore, there is definite evidence that epinephrine is involved in carbohydrate metabolism, both in the liver and in the muscle. It catalyzes the reaction, glycogen → glucose, and possibly, glucose → lactic acid.

The secretory activity of the adrenal medulla is intimately associated with the activity of other hormone-secreting organs. There is a close interrelationship between adrenal activity and the secretion of insulin by the pancreas glands. Both epinephrine and insulin influence the blood-sugar level. The activity of the pancreas increases with an increased epinephrine output, and *vice versa*, so that the two systems, while they supplement each other, may be regarded as mutually antagonistic. The secretory activity of other endocrine glands likewise appears to be influenced by the epinephrine level; in particular, complementary effects are indicated for the activity of the adrenal medulla and the posterior lobe of the pituitary.

In spite of the remarkable and powerful pharmacological and physiological effects which epinephrine exerts on the organism, it is rather surprising to note that the destruction of the medulla of both adrenals is not fatal, and indeed such destruction seems to have no particular effect provided that sufficient functional cortical tissue of the adrenals is left. The removal of both glands in their entirety is fatal. It therefore appears that the medulla is not essential to life and that therefore epinephrine is not an indispensable hormone. In view of this fact it would be anticipated that there are no recognized diseases due to hypomedullary adrenalism. Hyperfunction, however, is to be expected. In all cases of hyperfunction so far described medullary tumors have been involved. The major symptoms of hyperfunction are hypertension, high blood pressure, glycosuria, and other distressing effects.

The Hormones of the Adrenal Cortex.—The cortex of the adrenals arises from mesoblast cells which secrete a hormone which is essential to the maintenance of life. This hormone, *cortin*, was only recently prepared in the form of an active extract.[10] which enabled adrenalectomized animals to live and show normal body function. In man

[10] Swingle, W. W., and Pfiffner, J. J., Studies on the Adrenal Cortex, I-III, *Am. J. Physiol.*, 96: 153–163, 164–179, 180–190 (1931); The Preparation of Adrenal Cortical Hormone, *Endocrinology*, 15: 335–340 (1931); Hartman, F. A., Brownell, K. A., and Hartman, W. E., A Further Study of the Hormone of the Adrenal Cortex, *Am. J. Physiol.*, 95: 670–680 (1930); Pfiffner, J. J., Swingle, W. W., and Vars, H. M., The Cortical Hormone Requirement of the Adrenalectomized Dog, with Special Reference to a Method of Assay, *J. Biol. Chem.*, 104: 701–716 (1934).

a pathological condition of the adrenal cortex results in Addison's disease, characterized by anemia, loss of appetite, general languor and debility, feeble heart action, gastrointestinal irritability, and a peculiar and characteristic brownish, splotchy skin pigmentation. In its acute form Addison's disease is invariably fatal, but the symptoms can be rapidly alleviated and complete cure can be obtained by the administration of cortin. Although toxic effects from overdosage of cortin have not yet been produced in animals, hypercortico adrenalism of tumor origin appears to be well established in cases of cortical tumor. The effects are distinctly on sex behavior and sex characteristics. It is possible that this is related to the fact that the chemical structure of cortin shows a close relationship to the chemical structure of certain of the sex hormones. Adrenal cortex tumor in young and adolescent boys results in precocious growth and maleness. Sometimes the result is merely that of excessive muscular virility. After puberty the result is premature senility and early death. Rarely in adult males is there a definite development of "femaleness." In such instances the breasts enlarge and may even secrete milk. The testes atrophy; there is a loss of sex desire, and a female type of obesity may develop. In the female the predominating change is toward maleness. Before puberty in girls the individual may become fat and muscular with hair appearing early on the pubis and also on the face. The voice becomes coarsened, the uterus and ovaries atrophy, menstruation does not occur, although the external sex organs may show hypertrophy. Similar changes occur when the tumor begins after puberty, with the additional effect of atrophy of the breasts. Removal of tumors in both young girls and adults causes the individual to return to all normal sex characteristics within a few months, although the deep, masculine voice persists for the longest period.

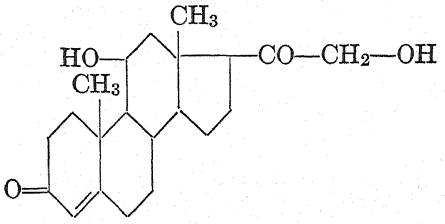
The chemical nature of cortin has been intensively investigated by Kendall,[11] Reichstein,[12] and Wintersteiner and Pfiffner.[13] It con-

[11] Kendall, E. C., *et al*, Isolation in Crystalline Form of the Hormone Essential to Life From the Suprarenal Cortex: Its Chemical Nature and Physiologic Properties, *Proc. Staff Meetings Mayo Clinic*, 9: 245–250 (1934); Concerning the Chemical Nature of the Hormone of the Adrenal Cortex, *ibid.*, 11: 351–352 (1936); The Chemistry of Crystalline Substances Isolated from the Suprarenal Gland, *J. Biol. Chem.*, 114: 613–631 (1936); and Chemical Studies of the Suprarenal Cortex II. The Identification of a Substance Which Possesses the Qualitative Action of Cortin; Its Conversion into a Diketone Closely Related to Androstenedione, *ibid.*, 116: 267–276 (1936).

[12] Reichstein, T., Über die Bestandteile der Nebennierenrinde, I-VI, IX, *Helv. Chim. Acta*, 19: 29–63, 223–225, 401–402, 402–412, 979–987, 1107–1126 (1936); 20: 817–827 (1937).

[13] Wintersteiner, O., and Pfiffner, J. J., Chemical Studies on the Adrenal Cortex II. Isolation of Several Physiologically Inactive Crystalline Compounds from Active Extracts, *J. Biol. Chem.*, 111: 599–612 (1935); and III. Isolation of Two New Physiologically Inactive Compounds, *ibid.*, 116: 291–305 (1936).

tains the sterol nucleus and is closely related structurally to the sex hormones (*vide infra*), and according to Reichstein [14] possesses the formula:



One of the special functions of cortin is the maintenance of the Na:K ratio in the blood and tissues and thus indirectly in the control of the blood volume. Adrenalectomized animals can survive normally if large amounts of sodium chloride are ingested. Verzar and Laszt [15] ascribe the effect of the cortical hormone on general bodily functions as lying in the necessity of this hormone for the formation and functioning of the riboflavinphosphoric acid of the yellow oxidation enzyme of Warburg and Christian. The phosphorylation process fails in the absence of the cortical hormone, as does the phosphorylation of fat and carbohydrates. Therefore the general metabolism of both fat and carbohydrate is seriously interfered with and deranged, and the general oxidative mechanism is broken down.

There is every evidence that cortin influences a great variety of bodily functions including the activity of many of the other glands of internal secretion. Evident correlations have been shown for kidney function [16] and for the gonad-pituitary system,[17] so that if and when

[14] Reichstein, T., Über Bestandteile der Nebennieren-Rinde, X, *Helv. Chim. Acta*, 20: 953–969 (1937); XI, *ibid.*, 20: 978–991 (1937).

[15] Verzar, F., and Laszt, L., Die Rolle von Lactoflavin und Flavinphosphorsäure bei Nebennierenrinden-Ausfall, sowie Jodessigsäurevergiftung, *Z. Vitaminforsch.*, 5: 265–275 (1936); Hemmung des Wachstums durch Jodessigsäure und antagonistische Beeinflussung durch Vitamin B_2 sowie Nebennierenrinden-Hormon, *Arch. ges. Physiol.*, 236: 693–704 (1935); Der Zusammenhang zwischen Vitamin B_2 und dem Hormon der Nebennierenrinde, *ibid.*, 237: 476–482 (1936); and Verzar, F., Adrenal Cortex and Intestinal Absorption, *Am. J. Digestive Diseases Nutrition*, 4: 545–546 (1937).

[16] Jimenez-Diaz, C., Death in Addison's Disease (Functional Renal Failure), *Lancet*, 231: 1135–1139 (1936).

[17] Davidson, C. S., and Moon, H. D., Effect of Adrenocorticotropic Extracts on Accessory Reproductive Organs of Castrate Rats, *Proc. Soc. Exptl. Biol. Med.*, 35: 281–282 (1936); Callow, R. K., and Parkes, A. S., The Occurrence of Oestrin and Progestin in Adrenal, Testis, and Hypophysis, *J. Physiol.*, 87: *Proc. Physiol. Soc.*, 28P–29P (1936); Casida, L. E., and Hellbaum, A. A., Ovarian Stimulation by Adrenal Extracts, *Endocrinology*, 18: 249–253 (1934); Allen, R., and Bourne, G., An Extract from the Adrenal Gland Causing Luteinisation of the Ovaries and Endometrial Hyperplasia, *Australian J. Exptl. Biol. Med. Sci.*, 14: 45–50 (1936); and Ehrenstein, M., and Britton, S. W., Further Observations on Cortico-adrenal Extracts, *Am. J. Physiol.*, 116: 42–43 (1936).

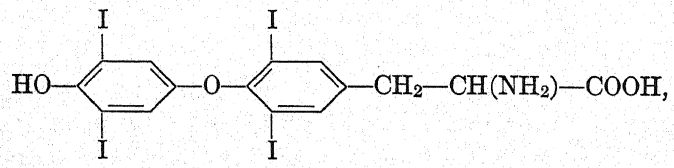
cortin is made available in adequate amounts to the physician, medicine will have at its disposal a therapeutic agent of the utmost importance.

The adrenal cortex contains large amounts of ascorbic acid[18] (vitamin C) suggesting that there may be some interrelationship between this vitamin and either the production or the functioning of the cortical hormones.

There is some evidence that the adrenal cortex contains at least two other hormones. One of these has been designated *cortipressin*.[19] This hormone, though apparently not identical with epinephrine, produces similar effects upon blood pressure, and its secretion by the cortical tissues probably accounts for the fact that animals survive the complete removal of the medullary adrenal tissue provided that sufficient cortical tissue is left. The hormone, like ephedrine, is effective when taken by mouth. Although concentrated extracts have been prepared, the pure hormone has not been identified.

The third hormone of the adrenal cortex has been designated *cortilactin*[20] and appears to influence the activity of the mammary glands. Nothing is known with regard to its chemical nature. In fact, it may be that the effect of the adrenals on mammary function is an indirect effect, the direct effect being on the pituitary and the secretion of prolactin by that organ.

THE HORMONES OF THE THYROID GLAND.—The thyroid is a divided endocrine gland situated on each side of the upper end of the trachea. The biochemistry of iodine in higher animals is largely, if not exclusively, associated with the hormone activities of the thyroid gland.[21] The secretions of the thyroid are the regulators of the metabolism of the body. They control both the basal and general oxidation levels of all cells concerned with oxidative metabolism. The specific chemical compounds identified with hormonal activity are thyroxine,



[18] Szent-Györgyi, A., On the Mechanism of Biological Oxidation and the Function of the Suprarenal Gland, *Science*, 72: 125–126 (1930); Identification of Vitamin C, *Nature*, 131: 225–226 (1933).

[19] Looney, J. M., and Darnell, M. C., The Blood Pressure-Raising Principle of Adrenal Cortex Extracts, *J. Biol. Chem.*, 114: *Proc. Am. Soc. Biol. Chem.*, lxii–lxiii (1936).

[20] Brownell, K. A., Lockwood, J. E., and Hartman, F. A., A Lactation Hormone of the Adrenal Cortex, *Proc. Soc. Exptl. Biol. Med.*, 30: 783–784 (1933).

[21] *Cf.* Harington, C. R., The Thyroid Gland, Its Chemistry and Physiology, Oxford University Press (1933).

discovered by Kendall in 1915 and synthesized by Harington in 1926, and 3.5-diiodotyrosine. Both these compounds have already been briefly considered in our earlier discussions of amino acids and important nitrogen bases. Thyroxine is a much more powerful metabolic stimulant than 3.5-diiodotyrosine, and it may be that the latter compound is simply a precursor or an intermediate in the formation of thyroxine. We have earlier commented on the unusual property of thyroxine, in that a single dose may maintain metabolic activities at an elevated rate for very extended periods of time, sometimes for several weeks.

That the thyroid gland contained iodine was discovered by Baumann [22] in 1895. In 1899, Oswald [23] showed that the iodine was bound in organic linkages and that an *iodothyroglobulin* could be extracted from the gland and that this iodothyroglobulin possessed the same physiological activity which was characteristic of the entire gland. Accordingly in speaking of the hormones of the thyroid gland it is necessary to include this iodoglobulin as the predominating hormone, although its activity is at least in part due to the presence of the unique amino acid, thyroxine, in the protein molecule. It is still uncertain in what form the thyroid hormone appears in the blood stream, whether as thyroxine, iodothyroglobulin, or as a peptide containing thyroxine and perhaps other iodine-containing organic compounds.

Aside from its effects upon oxidative metabolism the hormone exhibits major control over ossification processes and the normal functioning of the central nervous system and a measure of control over the development of the sex organs. Two general types of phenomena are associated with dysfunction of the thyroid: (*a*) in the absence of an adequate iodine supply or in a failure of the synthetic mechanisms within the gland, there is an inadequate secretion of the hormone; and (*b*) the synthetic and secretion mechanisms may become too greatly stimulated, resulting in an excessive secretion of the hormone and an overstimulation of metabolic activities. Diseases resulting from both hormonal insufficiency and hormonal excess are common.

Hormonal insufficiency resulting from a lack of iodine produces both simple goiter and cretinism. In simple goiter there may be a great enlargement of the thyroid gland, apparently a compensatory hypertrophy, more tissue being produced in the attempt to increase hormonal secretion. The body temperature is lowered, nervous symptoms appear, mental processes are dulled, weakness and lethargy become evident, the individual is easily exhausted, and unless the symptoms are relieved the patient progressively becomes worse until death

[22] Baumann, E., Über das normale Vorkommen von Jod im Thierkörper, *Z. physiol. Chem.*, 21: 319–330 (1895).

[23] Oswald, A., Die Eiweisskörper der Schilddrüse, *Z. physiol. Chem.*, 27: 14–49 (1899); Zur Kenntniss des Thyreoglobulins, *ibid.*, 32: 121–144 (1901).

ensues. The cretin is the extreme example of hormonal insufficiency, a congenital idiotic dwarf, usually born of a goiterous mother.

In adults hypothyroidism results in myxedema. Both conditions may be cured by either thyroxine or thyroid. Myxedema may be cured by 3.5-diiodotyrosine, given orally, although over *fifty times* the requisite dose of thyroxine is required, showing that it is not purely an iodine effect. Very definite symptoms of hypothyroidism may exist in adults without myxedema. These are always associated with a low basal metabolic rate. The symptoms are a tired, worn-out feeling, undue fatigability, loss of strength, nervousness, vague pains, sensitiveness to cold and infections, and sterility.

Hyperthyroidism apparently results from two types of pathological goiter, Graves' disease which is frequently (apparently inaccurately) designated exophthalmic goiter, involving diffuse hyperplasia (hypertrophy), and a non-hyperplastic goiter caused by a localized adenoma. The medical profession speaks of both types as toxic goiter. An excess of anterior pituitary thyrotropic factor is believed by some authorities to be involved in the hyperthyroidism. It has been known for some time that various iodine compounds, especially Lugol's solution (iodine in KI), are effective in the treatment of toxic goiters, particularly pre-operative, but the cause of the beneficial action is not known.

THE HORMONES OF THE PARATHYROID GLANDS.—The parathyroid glands are small (3-15 mm. by 2-3 mm.) compact glands composed of epithelial cells, usually but not invariably situated adjacent to the dorsal surface of the thyroids. The French physiologist, Gley, in 1891, first demonstrated that the removal of the parathyroid glands resulted in tetany and death. In 1909, MacCallum and Voegtlin [24] found that the removal of the glands was accompanied by a fall in blood calcium to about one-half its normal level and that tetany and death could be prevented by the injection of calcium salts at suitable intervals. It was this finding that led to the theory that the function of the parathyroids is to regulate the calcium metabolism of animals. It has since been shown that sufficient dietary calcium can prevent the results of parathyroidectomy. The rat does not seem to be as dependent as other animals on the parathyroid and accordingly can be used in experimental studies of chronic hypoparathyroidism. Parathyroidectomy in rats results in the development of opaque, brittle, distorted teeth and decalcified bones. In 1924, Hanson [25] and

[24] MacCallum, W. G., and Voegtlin, C., On the Relation of Tetany to the Parathyroid Glands and to Calcium Metabolism, *J. Exptl. Med.*, 11: 118–151 (1909).

[25] Hanson, A. M., The Hormone of the Parathyroid Gland, *Proc. Soc. Exptl. Biol. Med.*, 22: 560–561 (1925).

Collip[26] independently prepared active hormone extracts from the glands. However, although we now have a very clear picture of the physiological role[27] played by the parathyroids and although standardized extracts are now available, little is known about the chemistry of the hormone. It is commonly called *parathormone*. It appears to be a protein. It gives the usual protein color tests. It is isoelectric at *p*H 4.8–4.9 and soluble in solutions more acid than *p*H 4.6 or more alkaline than *p*H 5.2. It is precipitated from solution by half-saturation with ammonium sulfate or full-saturation with sodium chloride. It does not dialyze through collodion membranes; it is destroyed by boiling with 10 per cent hydrochloric acid or 5 per cent sodium hydroxide for an hour; and its activity is destroyed by either peptic or tryptic digestion. Accordingly the hormone is not effective when given by mouth. The most active preparations contain about 15.5 per cent of nitrogen. It has not been obtained in crystalline form.

Physiologically the hormone seems to exert some direct or indirect action on the labile mineral structures of the skeleton. Either hypo- or hyperparathyroid function results in a decalcification of the skeleton. Clinical hypoparathyroidism occurs most frequently after thyroidectomies where care has not been taken to preserve intact the parathyroid tissue. Parathyroid extracts have proved effective in certain cases of tetany in children and in other cases involving chronic convulsions, irrationalism, acute maniac excitation, etc., so that there is a possibility that hypoparathyroidism may be involved in certain instances in such conditions. Clinical hyperparathyroidism is demonstrated at its extreme in generalized *osteitis fibrosa* (von Recklinghausen's disease of bone) which was first described in 1891 but has only recently been definitely associated with the parathyroids. This disease appears almost invariably to result from a parathyroid tumor, and the removal of the tumor, which was first successfully accomplished in 1926, results in a return to normal. The disease can be simulated experimentally in animals by overdosing with parathyroid extracts. The skeleton is extensively decalcified, the bones no longer throw a dense X-ray shadow, hypercalcemia results, and there is a marked excretion of both calcium and phosphorus. Hyperparathyroidism appears to be invariably the result of a marked hypertrophy of the gland.

Another disease which is possibly due to chronic hyperparathy-

[26] Collip, J. B., The Extraction of a Parathyroid Hormone Which Will Prevent or Control Parathyroid Tetany and Which Regulates the Level of Blood Calcium, *J. Biol. Chem.*, 63: 395–438 (1925); Collip, J. B., Clark, E. P., and Scott, J. W., The Effect of a Parathyroid Hormone on Normal Animals, *ibid.*, 63: 439–460 (1925); Collip, J. B., and Clark, E. P., Further Studies on the Parathyroid Hormone, *ibid.*, 66: 133–137 (1925).

[27] Thomson, D. L., and Collip, J. B., The Parathyroid Glands, *Physiol. Rev.*, 12: 309–383 (1932).

roidism is known as the "marble bone disease." In this disease the bones become exceedingly brittle. The effect of the parathyroid hormone on calcium metabolism has led to attempts to associate the action of the hormone with the effects of vitamin D, but no such interrelationship has been established.

Potent parathyroid extracts have been employed to mobilize lead and remove it from the body in cases of chronic lead poisoning. The lead, along with calcium, is largely deposited in the skeleton, probably as the very insoluble tertiary lead phosphate. The administration of the parathyroid hormone, bringing about, as it does, a bone decalcification, also liberates lead so that the amount of lead secreted in the urine is greatly increased.

THE HORMONE OF THE THYMUS GLAND.—In most animals the thymus gland is situated in the upper portion of the thorax, although in some it is found in the neck. In the phylogenetic scale it appears first in the fishes. It is lacking in *Amphioxus*. It usually is of a grayish red color and generally consists of two lobes joined across a median plane. Each lobe is divisible into a cortex and a medulla, the latter containing characteristic concentric structures known as "Hassall's corpuscles." In man the gland normally reaches its maximum development in early childhood, during the period of most rapid body-growth, and then recedes in size, atrophying after puberty. In castrates it may persist for much longer periods.

The hormone of the thymus gland appears to speed up cell proliferation and cell growth of all tissues, and therefore influences the growth rate from birth to puberty. However, it seems to have no effect once normal adult growth has been attained.

The first extract to be made which exerted growth-promoting properties when injected into growing rats was made by Asher [28] in 1930. He called the concentrated purified extract *thymocrescin*. The product was stated to be free from protein and lipids. Asher regarded it as a sulfur-containing polypeptide. General growth, growth of the skeleton, and growth of the gonads all appeared to be accelerated by thymocrescin.

[28] Asher, L., Der Einfluss der Thymus auf das Wachstum und die Herstellung eines wirksamen Thymusstoffes Thymocresin, *Endokrinol.*, 7: 321–327 (1930); *cf.* also Asher, L., and Stotzer, P., Beiträge zur Physiologie der Drüsen, 124. Fortgesetzte Untersuchungen über die Wirkungsweise des Thymocresins, *Biochem. Z.*, 234: 1–18 (1931); Nowinski, V. W., Fortgesetzte Untersuchungen über Einfluss des Thymocrescins auf das Wachstum, *ibid.*, 249: 421–423 (1932); Asher, L., and Zenklusen, A., Beiträge zur Physiologie der Drüsen. 128. Fortgesetzte Untersuchungen über Thymocrescin, *ibid.*, 252: 309–324 (1932); Röthlisberger, P., Vergleichende Untersuchungen über den Einfluss von Thymocrescin und Lymphdrüsenextrakten auf das Wachstum, *ibid.*, 253: 137–142 (1932); and Asher, Doris, Weitere Isolierung des wachstumfördernden Thymocrescins, *ibid.*, 257: 209–212 (1933).

In 1930, Hanson prepared a thymus extract by an entirely different method which was not extensively tested until Rowntree [29] began his series of experiments three years later, using Hanson's original extracts. This extract and similar ones, which have shown such remarkable effects in Rowntree's hands, were so heterogeneous and impure that analyses give no hint as to the chemical nature of the hormone. Beginning with young rats, 1 cc. of extract was given daily by intraperitoneal injection until maturity and through gestation and lactation of the females. The young rats of the next generation were treated similarly beginning on the sixteenth to twentieth day of life. This was continued through successive generations. Precocity began to appear in the young born in the later litters of the F_1 generation, and continued to be greater in succeeding generations so that by the F_9 generation the physical, sexual, and psychic development was extraordinarily precocious. Representative data are shown in Table LXXXII.

TABLE LXXXII

APPROXIMATELY AVERAGE EFFECT OF INJECTIONS OF THYMUS HORMONE ON PHYSICAL CHARACTERISTICS OF RAT PROGENY
(DATA OF ROWNTREE, *et al.*)

	Normal Control Rats	Effect of Thymus Extracts on Treated Rats		
		F_1 Generation	F_8 Generation	F_9 Generation
Weight at birth	4.6 gm.	5.1 gm.	6.5 gm.	6.0 gm.
Ears opened	3 days	2½ days	Birth	Birth
Teeth eruption	9 "	8½ "	Birth	Birth
Hair appeared	14 "	11 "	1 day	1 day
Eyes opened	15 "	13 "	2 days	1½ days
Testes descended	37 "	22 "	3½ "	2½ "
Vagina opened	60 "	40 "	17 "	6 "
Pregnant	80 "	70 "	22 "	
First litter	102 "	92 "	43 "	

After the fifth generation the young rats ran about the cage when only 2–3 days of age, being as alert as normal rats at 16–20 days of age. Weaning is possible at 48 hours, after which they can nest for

[29] Rowntree, L. G., Clark, J. H., Hanson, A. M., and Steinberg, A., The Biologic Effects of Thymus Extract (Hanson), *J. Am. Med. Assoc.*, 103: 1425–1430 (1934); Biologic Effects of Thymus Extract (Hanson), *Arch. Internal Med.*, 56: 1–29 (1935); Rowntree, L. G., Clark, J. H., Steinberg, A., Hanson, A. M., Einhorn, N. H., and Shannon, W. A., Further Studies on the Thymus and Pineal Glands, *Ann. Internal Med.*, 9: 359–375 (1935); Rowntree, L. G., The Thymus Gland, *J. Am. Med. Assoc.*, 105: 592–595 (1935).

themselves, and need no further parental care. They can swim at the third day. These rats do not become larger than normal at maturity, but reach maturity in weight and size in about one-half normal time. The growth-rate acceleration reached its maximum in the F_4 generation. The rats are reported to be more fertile than normal.

In a recent brief note in Science Service, Rowntree [30] reports that preliminary experiments with injection of glutathione produce an effect similar to that produced by the thymus extract. Glutathione was present in appreciable amounts in the potent thymus extract. If this proves to be correct, it will identify the first of the more complex protein-like hormones. Incidentally, a sulfur-containing polypeptide was originally postulated by Asher.

There are no known clinical diseases of hypo- or hyperthymus activity. Thymectomy in five successive generations of rats has produced retarded growth, which can be overcome by thymus therapy.

THE HORMONES OF THE PINEAL GLAND.—The pineal gland is a small, flattened, pine-cone-shaped body, an outgrowth of the epithalamus, situated in the mid-brain. It contains neurologia and ependymal cells, the latter probably being the secretory cells.

Feeding pineal tissue [31] to tadpoles causes them to become so translucent that the internal organs are rendered visible. The translucency develops about 30 minutes after feeding on the tissue and persists for several hours.

Hanson, at Rowntree's suggestion, prepared an extract of the pineal gland which Rowntree *et al.*[32] found to be physiologically potent when injected in a manner similar to that in the earlier thymus studies.

Practically nothing is yet known regarding the chemical nature of the active principle or principles. Inasmuch as Hanson's aqueous extracts (acidified) yielded an apparently active picrate, the chemistry points to a protein-like rather than lipid-like substance.

[30] *Science*, 84: Supplement, p. 9 (Dec. 4, 1936).

[31] Huxley, J. S., and Hogben, L. T., Experiments on Amphibian Metamorphosis and Pigment Responses in Relation to Internal Secretions, *Proc. Roy. Soc.* (London), 93B: 36–53 (1922); and McCord, C. P., and Allen, F. P., Evidences Associating Pineal Gland Function with Alterations in Pigmentation, *J. Exptl. Zool.*, 23: 207–224 (1917).

[32] Rowntree, L. G., Clark, J. H., Steinberg, A., Hanson, A. M., Einhorn, N. H., and Shannon, W. A., Further Studies on the Thymus and Pineal Glands, *Ann. Internal Med.*, 9: 359–375 (1935); Rowntree, L. G., Clark, J. H., Steinberg, A., and Hanson, A. M., Biological Effects of Pineal Extract (Hanson). Amplification of Effects on the Young Resulting from Treatment of Successive Generations of Parent Rats, *J. Am. Med. Assoc.*, 106: 370–373 (1936); and Rowntree, L. G., Clark, J. H., Steinberg, A., and Hanson, A. M., The Biologic Effects of Pineal Extract (Hanson), *Science*, 83: 164–165 (1936).

Rowntree's injection experiments with rats, using solutions of the picrate, indicate a stimulation by it of the gonadotropic hormone of the anterior pituitary. The general effect on the rats in succeeding generations was the production of sexually precocious dwarfs. Growth, in general, was suppressed, the rats being only one-third or less as large as normals at corresponding age in the growth period, but the onset of adolescence and sexual maturity was greatly accelerated. In the F_4 generation the teeth erupted at 4 days, fur appeared at 5 days, the eyes opened at 6 days, the testes descended at 5 days, and the vagina opened at 24 days of age instead of the normal ages, as shown in Table LXXXII.

Rowntree reports no abnormalities in four successive generations of *pinealectomized* rats. Clear-cut clinical evidence of hypopineal function is lacking. A few cases of possible hyperpineal function, associated with tumorous condition of the gland, have been reported in young children, usually boys. There is abnormal growth, premature genital development, and early death.

THE HORMONE OF THE PANCREAS GLAND.—The pancreas gland is a large, elongated gland located below the stomach between the spleen and duodenum. The direct secretion of the gland, the pancreatic juice containing proteolytic enzymes, passes through the pancreatic duct into the duodenum. The gland contains areas composed of cells, smaller than the normal pancreas secretory cells. These areas, known as the *islets of Langerhans,* give rise to the hormone *insulin,* which passes into the blood circulation and not into the pancreatic duct. The islets comprise only 1/30–1/100 of the mass of the pancreas.

The relation of the pancreas to diabetes was shown first by von Mering and Minkowski, in 1889, through experimental pancreectomy in dogs. It was soon shown, however, by Hédon (1892), Minkowski (1892), and Gley and Thiroloix (1892), working independently, that the relation of the pancreas to the sugar metabolism was through an internal secretion. They succeeded in keeping pancreectomized dogs in normal condition by grafting the pancreas under the skin, thus removing all possibility of its nerve connections being related to its activity. After this discovery many attempts were made to prepare active extracts of the gland. It remained for Banting, Best, Macleod, and Collip [33] to prepare the first successful extract in 1921. This discovery was recognized by the award of the Nobel prize to Banting and Macleod in 1923. They named the hormone *insulin.* Although insulin does not cure diabetes, by its use diabetic patients are able

[33] Banting, F. G., and Best, C. H., The Internal Secretion of the Pancreas, *J. Lab. Clin. Med.*, 7: 251–266, 464–472 (1922); Banting, F. G., Best, C. H., Hepburn, J., Collip, J. B., Macleod, J. J. R., and Noble, E. C., Studies on Experimental Diabetes, III-VI, *Trans. Roy. Soc. Canada,* [3] 16 (Sect. V): 27–29, 31–33, 35–37, 39–41 (1922).

to have a fairly normal existence. Mathews states that considerably over 1,000,000 people in the United States have diabetes at the present time.

Insulin was isolated as a crystalline protein of low particle weight (35,100) by Abel [34] in 1926. The bulk of the molecule is accounted for [35] by eight amino acids, 30 per cent leucine, 21 per cent glutamic acid, 12 per cent cystine, 12 per cent tyrosine, 8 per cent histidine, 3 per cent arginine, 2 per cent lysine, and about 10 per cent of proline. About 0.5 per cent of all naturally crystalline insulin consists of zinc.[36] The zinc can be replaced by cadmium or cobalt. About three metal ions are associated with each insulin molecule. Zinc seems to have a stabilizing effect, for zinc-free insulin slowly decomposes on incubation at 52° C., which does not occur when zinc is present or added.

Insulin crystallizes in a rhombohedral cell with hexagonal axes of $a=74.7$ Å and $c=30.2$ Å. In the cell each molecule is surrounded by eight others, two at a distance of 30.2 Å and six at a distance of 44.3 Å. It is isoelectric at *p*H 5.5-5.6, and is free from phosphorus. It contains 3.2 per cent of sulfur, and its physiological activity appears to be closely associated with the sulfur-containing (cystine ?) portion of the molecule. Reducing agents destroy the physiological activity, which is not regained on subsequent oxidation. Inactivation parallels the liberation of —SH groups.

Miss Wrinch [37] has summarized the chemical, physical, and crystallographic data on insulin and has discussed these data with respect to her cyclol theory (*vide supra*). She concludes that the molecule is built on the cyclol pattern and contains 288 amino acid residues, the same number as is proposed [38] for egg albumin.

The protein nature of insulin predicts that it could not be especially effective, if at all, by mouth, inasmuch as it would be digested in the intestinal tract. A major development in insulin therapy during the past few years has been the preparation of compounds of insulin with protamines. Such compounds have already been noted in our

[34] Abel, J. J., Crystalline Insulin, *Proc. Natl. Acad. Sci. U. S.*, 12: 132–136 (1926).

[35] Jensen, H., and Evans, E. A., Jr., Die Einwirkung von Säure und Alkali auf Insulin, *Z. physiol. Chem.*, 209: 134–144 (1932); and Jensen, H., and Wintersteiner, O., Studies on Crystalline Insulin XVII. The Hydrolysis Products of Insulin, *J. Biol. Chem.*, 98: 281–287 (1932).

[36] Scott, D. A., Crystalline Insulin, *Biochem. J.*, 28: 1592–1602 (1934); Scott, D. A., and Fisher, A. M., Crystalline Insulin, *ibid.*, 29: 1048–1054 (1935); and Fisher, A. M., and Scott, D. A., Zinc Content of Bovine Pancreas, *ibid.*, 29: 1055–1058 (1935).

[37] Wrinch, D. M., On the Structure of Insulin, *Trans. Faraday Soc.*, 33: 1368–1380 (1937).

[38] Bergmann, M., and Niemann, C., On the Structure of Proteins: Cattle Hemoglobin, Egg Albumin, Cattle Fibrin, and Gelatin, *J. Biol. Chem.*, 118: 301–314 (1937).

consideration of the protamines. By use of these compounds the absorption of injected insulin from the tissues is slowed down, thereby rendering its action more uniform and more continuous.

The action of insulin is possibly limited to control of the reaction glucose → glycogen in the liver, heart, and the skeletal muscles. Obviously the control of this reaction will also control the removal of glucose from the blood stream. There is also a theory that insulin controls the formation of phosphoric acid esters of glucose, which is the first intermediate step in its oxidation. If glycogen is burned directly, as some believe, the control of the reaction, glucose → glycogen, is sufficient to explain the relation of insulin to diabetes.

Clinical hypoinsulinism, diabetes mellitus, seems to be associated with degeneration and atrophy of the cells of the islets of Langerhans which produce insulin. The blood sugar rises to highly abnormal levels, and both carbohydrate and fat metabolism are profoundly altered. The initial cause or causes of the dysfunction are not known.

Clinical hyperinsulinism is also known. This is frequently associated with tumor of the pancreas cells which produce insulin. The symptoms are essentially those of overdosage of insulin. In the order of onset they are: (1) fatigue and lassitude; (2) cold perspiration, palpitation, tremor, thirst, fear; (3) clouded senses, pseudo-alcoholic intoxication, hallucinations; (4) convulsions and paralysis, with loss of memory; (5) coma. The blood sugar falls to a negligible value. It is probable that most of the symptoms are traceable to disturbances arising in the central nervous system, an actual brain starvation, since the brain is dependent for its oxidative processes upon the blood sugar and has no store of available carbohydrate which can be drawn upon in an emergency.

THE HORMONES OF THE PITUITARY GLAND.—The pituitary is a small endocrine gland situated beneath the brain in the sella turcica of the sphenoid bone. In man it averages about 0.57 gram in weight. "No other single structure in the body is so doubly protected, so centrally placed, so well hidden." Abundant evidences are accumulating that it exerts a master control over a great variety of bodily functions, including the activities of the other endocrine glands. If one organ of the body can be said to be more important than another, that role must be ascribed to the pituitary.

The pituitary is commonly divided into two lobes, the anterior and the posterior, usually referred to as either the anterior or posterior pituitary, connected by the *pars intermedia*. In whales the *pars intermedia* is absent, and the two lobes are entirely separate. The anterior lobe or *pars distalis* is composed of both *chromophile* and *chromophobe* cells distributed somewhat indiscriminately throughout the gland. The posterior lobe, on the other hand, is composed of two distinct types of tissue, an inner core or *pars nervosa* and an outer lining of epithe-

lial cells, the *pars intermedia*. The small size of the gland and the interlacing of various types of tissue have made it difficult to decide definitely the particular type of tissue which gives rise to any particular hormone. Therefore a study of the hormones of the whale pituitary may assist in deciding some of the conflicting evidence.

There appears to be no question but that the pituitary secretes a multiplicity of hormones. The literature [39] is exceedingly voluminous and confusing with respect to the number of hormones, their physiological functions, and their physical properties. No pure crystalline hormone has as yet been isolated. The evidence all points toward hormones of protein or polypeptide nature, in many instances of an exceedingly labile nature so that the activity is lost in attempts to concentrate or purify the extracts. Earlier in this chapter we have listed nine hormones ascribed to the anterior pituitary and two ascribed to the posterior lobe. These do not represent all the principles for which claims are made in the literature, nor is there any certainty that still more potent substances do not remain to be discovered.

Because of the uncertain state of our *chemical* knowledge, only the briefest mention will be made of the individual pituitary hormones.

Posterior Pituitary Hormones.[40]—At the present time the evidence points to only two specific principles, although chemically similar principles appear to be present in the posterior lobe, which produce specific pharmacological effects. Not all endocrinologists accept them as true endocrine principles, secreted from the gland under physiological conditions. The two principles of the posterior lobe are (*a*) *pitocin* (*oxytocin*) (*alpha-hypophamine*) and (*b*) *pitressin* (*vasopressin*) (*beta-hypophamine*). Proprietary extracts of the posterior lobe, containing both principles, are available under the name pituitrin.

Pitocin is apparently a cystine-containing polypeptide which is rich in tyrosine. Pitressin seems to be similar, but richer in cystine.[41] The chief effect of pitocin is on smooth muscle. Concentrates have been made which are 1,000–1,250 times as powerful as is histamine.

[39] For reviews see Cameron, A. T., *loc. cit.*; Abel, J. J., Physiological, Chemical and Clinical Studies on Pituitary Principles, The Harvey Lectures, L. B. Lippincott Company, Philadelphia (1923–1924); Abel, J. J., The Chemistry and Function of the Pituitary Gland, *Ind. Eng. Chem.*, 16: 1031–1033 (1924); and Geiling, E. M. K., The Pituitary Body, *Physiol. Rev.*, 6: 62–123 (1926).

[40] Geiling, E. M. K., The Posterior Hypophysis, Chapter X, in Glandular Physiology and Therapy, A Symposium, American Medical Association, Chicago (1935).

[41] Gulland, J. M., The Oxytocic Hormone of the Posterior Lobe of the Pituitary Gland. II. The Action of Nitrous Acid and Nitric Acid, *Biochem. J.*, 27: 1218–1228 (1933); Gulland, J. M., and Macrae, T. F., III. The Action of Preparations of Plant Proteolytic Enzymes, *ibid.*, 27: 1237–1247 (1933); and Sealcock, R. R., and du Vigneaud, V., Studies on the Reduction of Pitressin and Pitocin with Cysteine, *J. Pharmacol.*, 54: 433–447 (1935).

It can be and is employed by obstetricians to stimulate contraction of the uterus, and apparently is normally involved in the termination of pregnancy.

Pitressin exerts several effects. One is on the blood capillaries, through which it affects blood pressure. Another is a pronounced antidiuretic effect. This effect seems to be brought about by increasing the reabsorption of water from the kidney tubules.

It would be anticipated that hypo- and hyperfunction of the posterior lobe would affect blood pressure, smooth-muscle contraction, and renal function. Such effects are, however, not common in human experience, nor are they readily produced in experimental animals. The only outstanding abnormal condition which is presumably associated with hypofunction of the posterior lobe is diabetes insipidus. This disease is characterized by the continued excretion of large volumes of a pale urine of low specific gravity, free from sugar. Normal kidney function is restored in many cases by continued injection of pituitrin, the effect being that of the pitressin present, the deficiency of which caused the diuresis. There have been some recent interesting suggestions that hypersecretion of pitressin may be one cause of gastric and intestinal ulcers, and that abnormal functioning of the pituitary is involved in the migrain-like "pituitary headache."

Anterior Pituitary Hormones.—This remarkable gland, weighing less than 0.5 gram in the human, produces no less than eleven different hormones, all but one of which may be regarded as exerting some specific effects. More than half of these may perhaps be regarded as "master" hormones, in that *they exert specific control either over other endocrine glands or over the production or secretion of other hormones!* Very little is known regarding the chemistry of any of the eleven accepted principles, but it seems safe to believe from the facts available that all are proteins or protein-like substances. The "master" hormones of the anterior pituitary are:

1. *The thyrotropic principle.* This hormone regulates the secretion of the active principle of the thyroid (thyroxine or thyroglobulin). The absence of this hormone produces all the effects of hypothyroidism; an excess produces all the effects of hyperthyroidism.

2. *The parathyrotropic principle.* This hormone regulates the proliferation of the parathyroid gland cells.

3. *Adrenotropic principle* (*interrenotropic principle*). This hormone controls the normal structure of the adrenal cortex and therefore indirectly controls the production of the adrenal cortical hormones.

4. *Pancreotropic principle.* This hormone has control over the islets of Langerhans and regulates either insulin production or insulin action.

5. *Prolan A.* This is a gametogenic hormone which stimulates male

germ cells, ova, and particularly the ovarian follicles and thus the production of their hormones.

6. *Prolan B.* This hormone, sometimes known as the "luteinizing hormone," stimulates the production of the corpus luteum cells of the ovaries and probably also the interstitial cells of the testes, and thus the production of their hormones.

7. *The A.P.L. (anterior pituitary-like) principle.* This hormone has an action similar to but not identical with that of prolan B. Its origin directly from the anterior pituitary is uncertain, but it is definitely found abundantly in the placenta. It is this principle which is also found abundantly in the urine during pregnancy in women and which furnishes the basis of the Aschheim-Zondek pregnancy test[42] (development of *corpora lutea* in immature mice by injection of ether-extracted urine). It is *not* found in the pregnancy urine of mice, rats, rabbits, bitches, cats, cows, sows, monkeys, or elephants.

8 and 9. The anterior pituitary produces two so-called *diabetogenic principles*, one known as the *blood-sugar-raising principle* and the other the *ketogenic principle.* The blood-sugar-raising principle exerts an antagonistic action towards insulin in the control of blood sugar, possibly acting on nerve centers which control carbohydrate metabolism. There is a strong possibility that the usual diabetes, diabetes mellitus, involves a deficiency of this principle as well as of insulin. The ketogenic principle stimulates the production of the so-called ketone bodies found in the urine in diabetes, especially β-hydroxybutyric acid. Other effects attributed to this principle are (1) the reduction of blood lipids, (2) a depression of the basal metabolic rate, (3) an increase in the specific dynamic effect of proteins.

10. *The growth hormone.*[43] Probably the anterior pituitary hormone which is capable of producing the most spectacular effects is the so-called growth-promoting hormone. Extracts containing, presumably, only this hormone, are sold commercially under various trade names, *e.g.*, antuitrin-G (growth), phyone.

The growth hormone seems to be produced by the alpha, acidophile, chromophile cells of the anterior pituitary which comprise about 37 per cent of the cell volume. Continued injection of preparations of this hormone into young animals was first shown by Evans, in 1921, to produce giants. His giant rats were essentially normal in proportion in all respects but corresponded in size to a human 10 to 12 feet tall. Similarly hyperproduction of this hormone in childhood produces human giants, several of which have been described in the medi-

[42] Aschheim, S., Pregnancy Tests, Chapter XVII, in Glandular Physiology and Therapy, A Symposium, Am. Med. Assoc., Chicago (1935).

[43] Evans, H. McL., The Growth Hormone of the Anterior Pituitary, Chapter III, in Glandular Physiology and Therapy, A Symposium, Am. Med. Assoc., Chicago (1935).

cal literature.[44] No naturally occurring pituitary giants have been described among animals, although some no doubt exist. A human hypophysial giant is described[45] as being 8 feet 4 inches tall and weighing 390 pounds at 19 years of age. He was normal in size at birth, but began to grow abnormally rapidly at once. He weighed 30 pounds at 6 months of age, 67 pounds at 18 months of age; at 5 years he was 5 feet 4 inches tall; and at 13 years he was 7½ feet tall. His height is reported to be still actively increasing. Pituitary giants are usually not physically normal in all respects. The joints are generally enlarged and awkwardly formed. The sexual development is usually subnormal, and may be expressed by either frigidity or sterility. They usually die before middle age, although there is a record of one attaining the age of 66.

Hyperproduction of growth hormone in adult humans results in the condition *acromegaly*, characterized by the gradual enlargement of the head and features and of the feet and hands, namely those parts of the skeleton which can enlarge. Pituitary giants gradually develop the acromegalic characteristics. Acromegaly has been simulated experimentally in dogs by injection of hormone extracts.

There is as yet no information as to whether the growth hormone acts directly on the tissues which it stimulates or indeed any inkling of how it acts. There is increased food consumption and definite indication of increased efficiency of food utilization, particularly of protein, but these effects are hardly sufficient to explain the remarkable results.

Hypofunction of the alpha cells results in dwarfs. Usually there is also hypofunction of the beta, basophile, chromophile cells, which comprise about 11 per cent of the anterior lobe volume. Inasmuch as these cells apparently produce the sex-controlling hormones of this organ, these hypopituitary dwarfs often remain infantile, and become excessively fat (Froelich's syndrome).

Hypofunction of the alpha cells together with hyperfunction of the beta cells produces sexually precocious dwarfs. Pituitary dwarfs develop well mentally and since the discovery of the growth hormone have been found to respond well to injection of growth-hormone preparations.

11. *Prolactin—the lactation hormones.* It is now clear that two distinct sets of hormones are involved in the lactation process. One set is required for the growth and development of the mammary gland; the other controls its secretory activity. Experimental work on both phases of this problem began about thirty years ago, but the actual solution had to await the isolation of the hormones involved. Early

[44] Cushing, H., The Pituitary Body and Its Disorders, J. B. Lippincott, Philadelphia (1912).

[45] Humberd, C. D., Giantism, Report of a Case. *J. Am. Med. Assoc.*, 108: 544–546 (1937).

studies on the relation of hormones to secretory activity of the mammary glands included extracts of the posterior pituitary (and later epinephrine itself), the corpus luteum, thymus, placenta, thyroid, testes, and the adrenals. The work eventually centered on the posterior pituitary as the source of the most important milk-secretion hormone. The positive effects which many of the earlier investigators obtained are now explained on the basis that epinephrine, because of its effect on the nervous system and blood pressure, merely causes a more complete discharge of the milk already in the gland and does not actually stimulate milk secretion. Interest was attracted to the anterior pituitary in 1928–1929 by the German workers, Grüter and Stricker,[46] who obtained the first evidence of a true lactation secretion hormone produced in this gland. Further definite evidence was obtained in 1930–1931 by Gardner and Turner,[47] who named it galactin. Riddle [48] isolated the hormone in a high state of purity in 1932 and named it prolactin, which name seems likely to be more generally adopted. The following facts are now fairly well established:

(*a*) The mammary glands develop under the influence of progesterone and the estrogenic hormones. Glands which are caused to develop in virgins, castrates, and males by injection of progesterone and estrone do not secrete milk but do so when prolactin is injected.

(*b*) Prolactin initiates and maintains lactation in the developed gland. In order to demonstrate the presence of this hormone the glands must first be properly developed through the action of progesterone and estrone. The mammary glands of castrated males may thus be developed and following the injections of prolactin will secrete milk.

(*c*) The ovarian hormones are antagonistic towards prolactin and prevent its secretion during pregnancy. At parturition there is sufficient decline in the ovarian hormone output to permit the secretion of prolactin. These relationships may explain the failure of lactation in certain individuals and suggest a probable clinical use for prolactin.

(*d*) The maintenance of lactation is also influenced by a nervous mechanism.

[46] Grüter, F., and Stricker, P., Über die Wirkung eines Hypophysenvorderlappenhormons auf die Auslösung der Milchsekretion, *Klin. Wochschr.*, 8 (II): 2322–2323 (1929).

[47] Gardner, W. U., and Turner, C. W., The Function, Assay and Preparation of Galactin—A Lactation Stimulating Hormone of the Anterior Pituitary and an Investigation of the Factors Responsible for the Control of Normal Lactation, Missouri Agr. Exp. Sta. Res. Bull. 196 (1933).

[48] Riddle, O., Bates, R. W., and Dykshorn, S. W., A New Hormone of the Anterior Pituitary, *Proc. Soc. Exptl. Biol. Med.*, 29: 1211–1212 (1932); The Preparation, Identification and Assay of Prolactin—A Hormone of the Anterior Pituitary, *Am. J. Physiol.*, 105: 191–216 (1933).

(*e*) There is some evidence that the adrenal cortex secretes a hormone which has been called *cortilactin* and which is of importance in milk production.

(*f*) Thyroxine has been shown to increase milk secretion in cows, especially at the close of lactation.

(*g*) Prolactin is secreted in the milk; this is true, at least, for women.

(*h*) Prolactin, like the other hormones of the anterior pituitary, is protein-like [49] in nature. It is completely destroyed by tryptic digestion. When salt-free it can be boiled for considerable periods of time without appreciable loss of activity.

(*i*) Prolactin, when injected into young virgin rats, has been shown by Riddle to stimulate the mothering, cuddling, and nesting instincts.

THE HORMONES OF THE ORGANS OF REPRODUCTION.—Several hormones already discussed have definite effects on sex characteristics or functions. This is true of thyroxine, the adrenal cortex hormones, the thymus and pineal principles, and certain specific principles of the anterior pituitary. The latter exert a direct effect on either the testes or ovaries, but at present it can only be surmised whether the thyroid, adrenal, thymus, and pineal principles act through the gonads or not. At any rate, we seem to have here the most outstanding example of the interlocking chain of structures which the glands of internal secretion exhibit.

Indirect evidence of hormones produced by the gonads existed prior to the isolation of the first of these in 1923 [50] from the ovarian follicular fluid. Since that time we have seen a most remarkable development in hormone chemistry. As a matter of fact, most of this development has come about within the past three or four years and was made possible by the discovery (1) that these hormones have the same structural nucleus as cholesterol and its related compounds, (2) that the various gonadal hormones are very closely related chemically, and (3) because of the advances in knowledge regarding the chemistry of the sterols in other laboratories, it has been a relatively simple matter to elucidate completely the chemical nature of these gonadal principles. The isolation of these hormones, the elucidation of their structural configuration, and in a number of instances their *synthesis* in the chemical laboratory must be looked upon as one of the major achievements of the organic chemist. Space will not permit an adequate review of this fascinating story. Many workers have contrib-

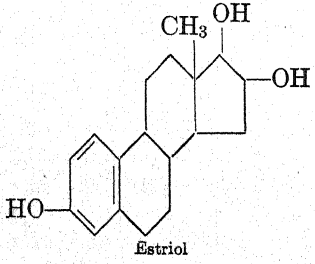
[49] Bates, R. W., Riddle, O., and Lahr, E. L., On the Protein Nature of Prolactin and of Follicle-Stimulating Hormones, *Proc. Soc. Exptl. Biol. Med.*, 31: 1223–1224 (1934).

[50] Allen, E., and Doisy, E. A., An Ovarian Hormone, *J. Am. Med. Assoc.*, 81: 819–821 (1923).

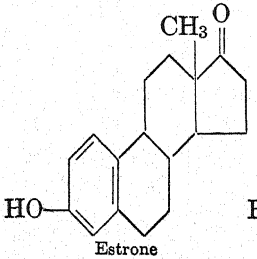
uted their mite. Among the more outstanding are Edgar Allen, Yale University; A. Butenandt, University of Göttingen; J. B. Collip, McGill University; E. C. Dodds, University of London; E. A. Doisy, Washington University, St. Louis; G. F. Marrian, University of Toronto; L. Ruzicka, Technische Hochschule, Zurich, and B. Zondek, Technische Hochschule, Berlin.

We have earlier commented upon the fact that the sterols, the bile acids, the saponins, the cardiac glycosides, the toad poisons, and the cortical hormone of the adrenal glands all are derivatives of the same fundamental nucleus. To this list we must now add the sex hormones and the carcinogenetic hydrocarbons (*vide infra*).

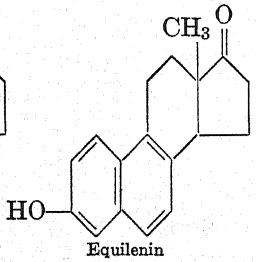
The Estrogenic Hormones.—At least five compounds are normally involved in the estrus mechanism. These are (1) estriol, (2) estrone, (3) equilenin, (4) equilin, and (5) estradiol, possessing the respective configurations noted in the formulas



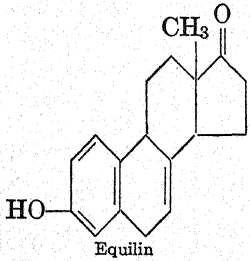
Estriol



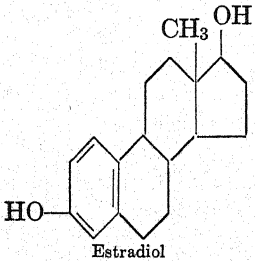
Estrone



Equilenin



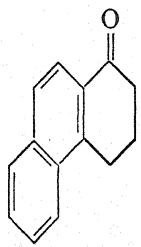
Equilin



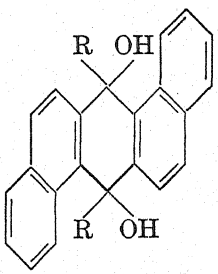
Estradiol

The knowledge of these configurations caused investigators to study the behavior of related compounds, and it was early found [51] that 1-keto-1.2.3.4-tetrahydrophenanthrene possessed estrogenic activity to a high degree, similar to the natural estriol, and estrone. These

[51] Cook, J. W., Dodds, E. C., Hewett, C. L., and Lawson, W., The Oestrogenic Activity of Some Condensed Ring Compounds in Relation to Their Other Biological Activities, *Proc. Roy. Soc.* (*London*), B114: 272–286 (1934).



1-Keto-1.2.3.4-tetrahydrophenanthrene



Dialkyldibenzanthracenediol

authors prepared an extensive series of related compounds. That series possessing the dialkyldibenzanthracenediol structure has been of outstanding interest. Here the type of alkyl radical in a large measure determines [52] the estrogenic activity of the compound. The dimethyl. di-*n*-amyl, and di-*n*-hexyl compounds are inactive. The diethyl and di-*n*-butyl compounds are active but considerably less so than the di-*n*-propyl compound. The diisopropyl compound was only about one-tenth as active as the *n*-propyl compound, and the isobutyl compound showed only about one-tenth the activity of the *n*-butyl compound. Dodds points out that the phenanthrene nucleus does not appear to be a necessity for estrogenic activity in synthetic compounds, for such compounds as 4.4′dihydroxydiphenyl, diphenyl-α-naphthylcarbinol, 1.2-dihydroxy-1.2-di-α-naphthylacenaphthene, and a number of related compounds are highly estrogenic. He suggests that the diphenyl nucleus or some similar arrangement of the benzene rings may be important.

All the estrogenic hormones arise in the follicular fluid of the ovaries, and apparently all occur together. Their secretion precedes ovulation. Estradiol is the most active and is the parent substance of those which were first isolated. The activity of these substances is progressively lost through either loss or addition of alcohol groups, or through further unsaturation of the ring structure (estradiol > estrone > estriol > equilin > equilenin.

Estradiol, estrone, and estriol control the occurrence of estrus in all species which show this physiological phenomenon and will also produce menstruation and its attending changes in women and primates which have undergone ovariectomy. Estrone, especially, has found clinical use in alleviation of severe subjective symptoms in the menopause in women. These hormones are active when taken by mouth.

The estrogenic hormones have a definite relation to the growth of

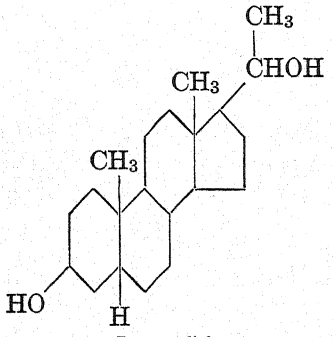
[52] Dodds, E. C., Chemical Structure in Relationship to Hormone and Biological Activity, *Helv. Chim. Acta*, 19 (*Fasciculus Extraordinarius*): E49–E57 (1936).

the mammary glands, especially the ducts and alveoli. They therefore play a specific role in the development of the glands during the attainment of sexual maturity.

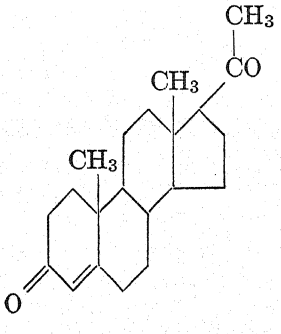
Equilin and equilenin may be regarded as derivatives of estrone (theelin). They occur in the urine of pregnant mares and exert only slight estrogenic activity. They perhaps merely represent a mode of inactivation of the estrone which persists during pregnancy and thus account for the failure of the animal to show estrus during pregnancy.

Estradiol, estriol, and estrone combine in the body with glycuronic acid where the acid is esterified on the hydroxyl groups of the hormones, to form *emmenins*. These apparently occur abundantly in the placenta, and, as would be expected, in pregnancy urine. The esterification modifies their action so that they have no estrogenic effect on castrates or on mature normal animals. The emmenins do produce estrus in normal sexually immature animals. They are effective by mouth as well as by injection.

The Hormones of the Corpus Luteum and Pregnancy.—Two compounds, *pregnandiol* and *progesterone*, closely allied in structure to the



Pregnandiol



Progesterone

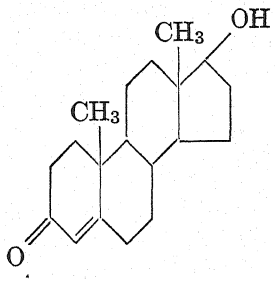
other sex hormones occur abundantly in the corpus luteum and in pregnancy urine. Some endocrinologists believe that they also are produced in the placenta. The active agent of this pair, progesterone (also called progestin, corporin, luteosterone, lutin), has the special functions of (1) inhibiting ovulation, (2) stimulating the growth of the uterus, and (3) sensitizing the uterus for the implantation of the embryo (or embryos). The absence of this hormone through the removal of the corpus luteum during the earlier part of pregnancy causes failure of implantation of the embryo, or, if the embryo is already implanted, it causes destruction of the pregnancy.

So far as is known the corpus luteum has no useful action except in pregnancy. It is not unanimously accepted, however, that its hormone is absolutely essential for the maintenance of pregnancy in all

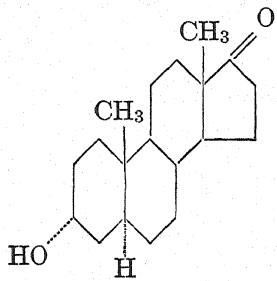
species, even in humans. Progesterone is not effective by mouth, but must be injected, usually subcutaneously, in oil. Progesterone, along with the estrogenic hormones, causes the development of the mammary gland in pregnancy. It appears to be the chief agent concerned with the preparation of these glands for lactation.

The Male Sex Hormones.—The male sex hormone, *testosterone,* and its relatively feeble derivative, *androsterone,* are remarkably closely related to the ovarian estrogenic hormones.

Testosterone differs from estradiol only in having an additional $-CH_3$ group and a ketone group in place of one of the —OH groups. Because of these differences there is, of course, less unsaturation in



Testosterone



Androsterone

the ring nucleus. These slight differences suggest the possibility of interconversion. In fact, interconversion does occur, and their closely related structure explains the apparent anomalous demonstration of Zondek that the materials richest in estrogenic substances are stallions' testes and urine, where both estradiol and estrone are found. The reverse conversion occurs in females, although not so extensively. Mathews [53] believes that the formation of relative excess of testosterone or estradiol, respectively, in early embryonic life determines the sex of the embryo. In the human this would have to occur before the third week, at which time the sex may be distinguished. Mathews emphasizes the fact that in many particulars every individual not only starts as an hermaphrodite but remains one at all stages of its life. This appears to be the case so far as the ovarian and testicular hormones are concerned. His idea is that a slight overbalance of male or female hormone starts the individual in the direction of male or female, which is kept going in that direction by the formation of the glands which produce the male or female hormone predominantly. In support of this he cites the experiment of Koch and Willier, who produced an ovary out of a left protestis in the developing chick embryo by injecting estrone into the incubating egg. In fowls the left protestis

[53] Mathews, A. P., Principles of Biochemistry, William Wood and Company, Baltimore (1936).

develops into the functional ovary of the hen, both sides developing testes in males. Thus, according to Mathews' reasoning, the so-called genital ridge of the mesoblastic tissue of the chick embryo from which testes and ovaries develop is hermaphroditic, not neutral, and needs only the predominance of male or female hormone to develop into male or female tissue. Mathews also speculates as to the possibility that the chromosome theory of sex determination is likewise explainable on the basis of predominance of male and female hormone.

The testicular hormone is very similar in structure to the corpus luteum hormones, pregnandiol and progesterone. There is definite evidence that testosterone is changed to progesterone in the stallion because the latter occurs in stallion urine. Another striking example either of specific function of male hormone in the opposite sex or its change to the female hormone is seen in the use of the female bittering as a test animal for the male hormone. The addition of testosterone or androsterone to the aquarium water in which the female bittering is to be placed causes growth of its ovipositor. This is not caused by addition of estriol or estrone.

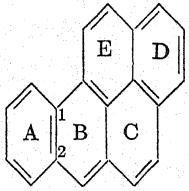
Testosterone and androsterone are produced in the interstitial tissue of the testes. The functions of these hormones are (*a*) the determination and control of all the secondary sex characteristics of males, and (*b*) the maintenance of the normal functional condition of the male accessory sex organs, especially the prostate and seminal vesicles. The control exercised over the secondary sex characteristics is manifested by its ability to promote comb growth and male plumage in cockerels and capons, the former being employed for biological assay. The male hormones have been shown to maintain the normal viability and mobility of spermatozoa, and the normal chemical and biochemical properties of the semen. The prostate degenerates in the absence of these hormones and is restored by their injection. This is also true of seminal vesicles. It should be remembered, however, that the production of these hormones is under the control of prolan B of the anterior pituitary. Simple prostate hypertrophy appears to be caused by unbalance between this gonadotropic hormone of the pituitary and the testicular hormones. When the testicular hormones are deficient, the hypophysis produces an excess of prolan B which in turn causes an excessive stirring of the interstitial cells to produce testosterone. The excess testosterone causes the prostate to enlarge.

Secretin.—Bayliss and Starling (*loc. cit.*) applied the term hormone to a secretion of the duodenal mucosa which induced a flow of pancreatic juice. Pavlov had earlier observed that acids in the duodenum would cause such a flow, but Bayliss and Starling showed that the action was not a nerve response and were able to prepare from duodenal mucosa an extract which, when injected into the blood stream, activated the pancreas and caused a copious flow of pancreatic

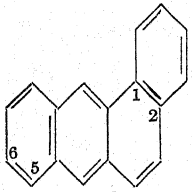
juice. They named this hormone *secretin*, and showed that it was stable to heat but was destroyed by oxidizing agents or by proteolytic enzymes. Secretin is insoluble in the usual organic solvents, dialyzes through collodion or parchment membranes, contains sulfur, is precipitated by picric, tannic, phosphotungstic, and trichloroacetic acids, and the weight of evidence is that it is a polypeptide or a proteose, although tyrosine, tryptophane, histidine, and phenylalanine are absent. Preparations which will induce secretion of pancreatic juice when as little as 0.02 mg. per kilo are injected have been prepared,[54] but these are recognized as still being impure.

THE CARCINOGENETIC HYDROCARBONS

It has been known for a number of years that repeated application of coal tar to the skin will eventually induce cancerous growths. Cook [55] and collaborators at the Research Institute of the Cancer Hospital, London, have been especially active in the investigation of this phenomenon. In 1933, they [56] reported the isolation and identification of the active constituent in coal tar. This proved to be 1.2-benzpyrene. 1.2-Benzanthracene was also isolated from the tar, and, although it is not itself markedly carcinogenetic, a number of its derivatives have been shown by Cook *et al.* to have great carcinogenetic



1.2-Benzpyrene



1.2-Benzanthracene

activity. It can be regarded as the parent substance of a host of carcinogenetic compounds. Substitutions in the 5- and 6-positions are especially potent, with substitutions in position-5 having in general somewhat greater carcinogenetic activity than similar substitutions in

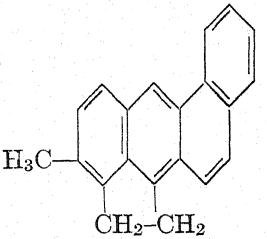
[54] Dale, H. H., and Laidlaw, P. P., A Method of Preparing Secretin, *Proc. Physiol. Soc.*, 1912, *J. Physiol.*, 44: xi-xii (1912); Ivy, A. C., Kloster, G., Drewyer, G. E., and Lueth, H. C., The Preparation of a Secretin Concentrate, *Am. J. Physiol.*, 95: 35–39 (1930); Ivy, A. C., The Role of the Hormones in Digestion, *Physiol. Rev.*, 10: 282–335 (1930); Still, E. U., Studies on the Physiology of Secretin I. On the Preparation and Isolation, *Am. J. Physiol.*, 91: 405–408 (1930); Secretin, *Physiol. Rev.*, 11: 328–357 (1931).

[55] Cook, J. W., *et al.*; see numerous papers in *Proc. Roy. Soc.* (*London*), *Series B*, and *J. Chem. Soc.*, 1930 to date.

[56] Cook, J. W., Hewett, C. L., and Hieger, I., The Isolation of a Cancer-Producing Hydrocarbon from Coal Tar, I, II, and III, *J. Chem. Soc.*, 1933: 395–405.

position-6. Substitutions in other positions do not appear to affect carcinogenetic activity greatly.

Among the derivatives having powerful carcinogenetic power is methylcholanthrene, which can be considered as a 5.6-dialkyl-1.2-benzanthracene and which bears close structural relationships to the sterols, the bile acids, and the sex hormones. Although the thesis has not been proved, it seems probable that methylcholanthrene or certain of its potent derivatives may arise in the body from the sterols, the bile acids, or the sex hormones, and thus be the normal carcinogenetic agents.



Methylcholanthrene

The reader who wishes to pursue this interesting and important series of studies, with particular reference to the space relationships of the organic chemistry which is involved, is referred to the most excellent summary of Fieser.[57]

THE PLANT HORMONES

Whereas the term "hormone" was originally intended to designate only specific principles elaborated by the animal body and in particular by the endocrine glands, it has now been adopted by plant physiologists to indicate those special factors which greatly stimulate plant growth.[58] The work in this field really dates back to Charles Darwin, who, in 1881, in his book, "The Power of Movement in Plants," suggested that, "When seedlings are freely exposed to a lateral light, some influence is transmitted from the upper to the lower part, causing the latter to bend." This hypothesis was first proved experimentally by

[57] Fieser, L. F., The Chemistry of Natural Products Related to Phenanthrene, Chapter III, Cancer-Producing Hydrocarbons, pp. 81–110, Reinhold Publishing Corporation, New York (1936).

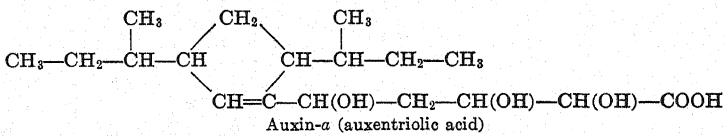
[58] For literature see Boysen-Jensen, P., "Growth Hormones in Plants," translated by G. S. Avery and P. R. Burkholder, McGraw-Hill Book Company, New York (1936). See also Koepfli, J. B., Thimann, K. V., and Went, F. W., Phytohormones: Structure and Physiological Activity. I, *J. Biol. Chem.*, 122: 763–780 (1938), where the literature with respect to chemical configuration and biological activity of 47 organic compounds is critically reviewed.

Boysen-Jensen, in 1910–1911, who blocked off the path of transmission with mica plates and thus demonstrated that something which promoted cell growth was being transmitted from the growing coleoptile tip toward the base of the coleoptile. These observations were followed by a host of investigations which finally culminated in the isolation and identification of the chemical structure of at least certain of the more important chemical compounds which are involved. In this series of studies the contributions from the laboratories of Kögl and Went, at the University of Utrecht, Boysen-Jensen, at Copenhagen, and Thimann, at the California Institute of Technology, should be especially recognized.

The method of testing for the presence of the growth-stimulating substances is either (*a*) to apply the material unilaterally directly to decapitated coleoptiles of *Avena sativa* or (*b*) to make up the material to be tested in a 3 per cent agar gel or in lanolin and apply these preparations, under standardized conditions, unilaterally to decapitated *Avena* coleoptiles. The curvature of the treated coleoptile after a standard period of time [110 minutes (Thimann), 120 minutes (Went), 3 hours (Boysen-Jensen)], depending upon the exact procedure which is being followed, is then measured. The curvature is proportional to the *concentration* of the growth substance in the material applied to the decapitated coleoptile.

Kögl named these growth substances, *auxins*, from the Greek αυξειν, to increase. Three compounds that show these remarkable growth-promoting properties have been isolated from natural sources, *i.e.*, *auxin-a*, *auxin-b*, and *heteroauxin*. All three are monobasic acids.

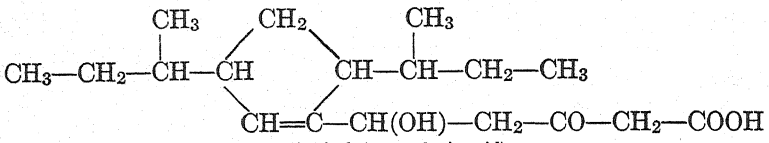
Auxin-*a* or *auxentriolic acid*, $C_{18}H_{32}O_5$, is a monocyclic trihydroxymonocarboxylic acid with one double bond and has the formula:



Auxin-*a* (auxentriolic acid)

It is thermostable, not decomposed by light, but undergoes slow spontaneous isomerization so that it becomes physiologically inactive after a few months, even when kept in the dark in a vacuum. It is relatively stable to acids but rather sensitive to alkalies.

Auxin-*b* or *auxenolonic acid* is closely related in structure to auxin-*a* but has only one hydroxyl group, the other hydroxyl groups of auxin-*a* being replaced by a carbonyl oxygen and hydrogen respectively. It likewise is heat and light stable, but is readily oxidized and becomes inactive in a few months, presumably through isomerization. *Heteroauxin* has proved to be *β-indoleacetic acid.*



Auxin-*b* (auxenolonic acid)

Both auxin-*a* and auxin-*b* have been obtained from malt and the oils of maize, mustard, sunflower, and flax. Maize oil is, in general, richer in auxins than are the other vegetable oils which have been examined. Kögl [59] *et al.* investigated thirty-six maize oils and found that oils from different geographical areas (and presumably maize sorts) differed widely in auxin content, the range being from 0–540 μg. per kilo with an average of 103 μg. Twenty-two samples of unmalted barley showed a range of 0–43 μg. auxin with an average of 5 μg. per kilo. Malting increased the auxin content up to the fifth day of malting when a maximum content of 420 μg. per kilo was observed. From one 16-kilo lot of maize oil, Kögl isolated 15 mg. of crude crystals of auxin-*a* and 15 mg. of crude crystalline auxin-*b*. On purification 6 mg. of pure auxin-*b* was obtained. One sample of maize oil was found which contained 10–13 mg. of auxin per kilo. From 160 kilos of this oil, Kögl isolated 238 mg. of auxin-*b* and 7 mg. of auxin-*a*. These data are given to indicate the enormous dilutions in which these growth substances occur in nature, and the difficulties which attended their isolation and identification.

Human urine was found by Kögl [60] to be a rich source of auxin-*a* and auxin-*b*, having an auxin content of approximately 2 mg. per liter. Urine also contains heteroauxin.[61] The relative growth-promoting activities of these three compounds are 50,000,000 AE [62] for both auxin-*a* and auxin-*b*, and 25,000,000 AE for heteroauxin (β-indoleacetic acid). Various synthetic compounds closely related to β-indoleacetic acid have been tested for growth-promoting properties, but none have been found which even approach the natural auxins in efficiency. β-(α-Methylindole)acetic acid has an activity of only 125,000 AE. β-In-

[59] Kögl, F., Erxleben, H., and Haagen-Smit, A. J., Über die Isolierung der Auxine a und b aus pflanzlichen Materialen. 9. Mitteilung über pflanzliche Wachstumsstoffe, *Z. physiol. Chem.*, 225: 215–229 (1934).

[60] Kögl, F., Haagen-Smit, A. J., and Erxleben, H., Über ein Phytohormon der Zellstreckung. Reindarstellung des Auxins aus menschlichen Harn, *Z. physiol. Chem.*, 214: 241–261 (1933); Studien über das Vorkommen von Auxinen im menschlichen und im tierischen Organismus, *ibid.*, 220: 137–161 (1933).

[61] Kögl, F., Haagen-Smit, A. J., and Erxleben, H., Über ein neues Auxin ("Heteroauxin") aus Harn, *Z. physiol. Chem.*, 228: 90–103 (1934).

[62] AE = "Avena Einheit," *i.e.*, that amount of growth substance which when present in a block of 3 per cent agar, 2 by 2 by 0.5 mm. in size, will in 2 hours cause a curvature of 10° in the decapitated coleoptile held at 22–23° C. in a relative humidity of 92 per cent.

dolepropionic acid, β-indolecarboxylic acid, β-2.3-dihydroindoleacetic acid, and β-(α-ethylindole)acetic acid were all inactive.

It is generally believed that the auxins are responsible for the growth of plant cells. Their function seems to be limited to cell enlargement, not to cell proliferation, although some workers have claimed that they speed up and facilitate cell division. They appear to be formed in the rapidly growing tips of the coleoptile and to be transported downward. They are also formed in root tips, buds, and apparently in the extremities of all rapidly growing plant parts. Heteroauxin has been isolated from various fungi, including yeasts, *Aspergillus niger,* and *Rhizopus* sp., and appears to be the principal, if not the sole, growth-promoting principle in these lower plant forms. Heteroauxin has already found a commercial application in inducing the formation of roots on cuttings, thereby making possible the commercial propagation of certain plants which normally could not be propagated in this manner.

In the preface to the first edition of this text it was emphasized that in the last analysis the chemistry of the cell is essentially the same both for plants and for animals. That this is true is becoming more and more evident as research progresses. Numerous workers are finding that the vitamins which hitherto have supposedly affected only animal processes are likewise playing important roles in the development of plants. Even certain of the sex hormones have been found to have a beneficial influence upon plant growth. Bonner [63] has summarized some of the more recent literature in this field, and his paper may be consulted for literature sources.

[63] Bonner, J., The Role of Vitamins in Plant Development, *Botan. Rev.*, 3: 616–640 (1937) (153 refs.); *cf.* also Robbins, W. J., Organisms Requiring Vitamin B_1, *Proc. Natl. Acad. Sci. U. S.*, 24: 53–56 (1938); Bonner, J., and Bonner, D., Ascorbic Acid and the Growth of Plant Embryos, *ibid.*, 24: 70–75 (1938); Robbins, W. J., and Kavanagh, F., The Specificity of Pyrimidine for Phycomyces Blakesleeanus, *ibid.*, 24: 141–145 (1938); The Specificity of Thiazole for Phycomyces Blakesleeanus, *ibid.*, 24: 145–147 (1938).

CHAPTER XXXVII

VITAMINS *

Vitamins may be defined as *organic substances which must be supplied in the diet of animals or may be synthesized in animals from essential dietary or metabolic precursors, and which exert a hormone-like or enzyme action in the control and coordination of specific chemical reactions in the animal body.* The vitamins are definite nutritive substances, although the quantitative requirements for them are relatively very small as in the case of some of the inorganic elements now recognized as essential for animal life. It is also an interesting fact that each of the recognized vitamins (with possibly one exception), like the hormones and enzymes, has been established by biological studies before its isolation and chemical identification.

The vitamins whose chemical structures have been established are vitamin A; thiamin chloride [aneurin or vitamin B (B_1)]; *l*-ascorbic acid (cevitamic acid or vitamin C); calciferol (vitamin D_2); *d*-riboflavin [the flavin factor or vitamin G (B_2)]; and nicotinic acid (P.-P., pellagra preventive vitamin). No name seems to have been proposed for vitamin A which would indicate its general chemical nature, although some name such as semi-β-carotenol or β-iononediisoprenol or β-ioninol or simply β-ionin might be as satisfactory as the names given to the other vitamins of known chemical identity.

Vitamins whose chemistry is not yet so clearly established as the above or for which biological evidence only is as yet available are numerous sterol derivatives having vitamin D action; vitamin E; a chick-antidermatitis factor, also called vitamin B_2 or filtrate factor or factor 2; the rat-antidermatitis (rat-antiacrodynia) vitamin (vitamin B_6 or factor 1, which has also been called vitamin H); vitamin B_4 (believed to be also an antiencephalomalacia factor for chicks); vitamin H (the anti-egg-white-poisoning factor, distinct from vitamin H, the rat antiacrodynia factor); vitamin K (an antihemorrhagic factor, necessary at least for chicks and humans); an antigizzard-erosion factor, not otherwise designated; and factor W (a supposed growth vitamin). Biochemists are debating whether proposed vitamins should continue to be so classed when they become identified as substances

* This chapter is contributed by Dr. Leroy S. Palmer, Professor of Agricultural Biochemistry in the University of Minnesota.

whose chemistry has long been known or already established in nutrition or in other fields of biochemistry. This applies to linoleic acid (which has been referred to as vitamin F) and citrin (a flavone or flavonol glycoside), first called vitamin P. There may be some justification for including citrin among the vitamins, for although its components have long been established chemically, they had not previously been known to play any role in animal life. The commercial exploitation of linoleic acid as a vitamin has been responsible, in part, for the biochemists' reluctance to accept a vitamin terminology for it. When the chemistry of all the vitamins is known and the alphabetical nomenclature abandoned in favor of specific names, linoleic acid will no doubt have to be included as a substance which, under certain conditions, exerts a vitamin effect according to the definition given above.

No attempt will be made here to describe the historical steps through which the vitamins were discovered. It will be sufficient to point out that two fields of work eventually combined into what has been referred to by McCollum as the newer knowledge of nutrition. These two fields of work were, first, a study of certain specific diseases, i.e., beriberi and scurvy, and second, attempts to nourish laboratory animals on synthetic diets composed of the known food components in the purified state.

The term "vitamine," from which our present word vitamin is derived, was coined by Funk [1] as the result of an attempt to isolate from food the substance, of as yet unknown nature, that prevented the disease beriberi. When beriberi thus came to be recognized as a "vitamine"-deficiency disease, the hypothesis was advanced that various other "vitamine"-deficiency diseases occur. At about the same time, laboratory studies in nutrition showed that natural foods contain chemical substances necessary for normal nutrition, but not classified under any of the ordinary groups of nutrients, *i.e.*, protein, carbohydrate, fat, and mineral salts. Of special importance was the discovery that these substances were incapable of chemical determination by the methods applicable to the nutrients mentioned. Particularly astonishing was the fact that the effect exerted by these accessory food ingredients was far greater than that shown by corresponding amounts of known nutrients and apparently quite out of proportion to the amounts evidently present in the food. The workers in the field of "vitamine"-deficiency diseases and those studying the strictly nutrition problems soon came to recognize that these new substances required for normal nutrition were apparently identical with the "vitamines," a lack of which was primarily concerned with the etiology of the deficiency diseases. It was in this way that these two fields of

[1] Funk, C., The Etiology of the Deficiency Diseases, *J. State Med.*, 20: 341–368 (1912).

investigation came together and contributed to the discovery of and the extension of our knowledge of vitamins. Advances in our knowledge of the vitamins at the present time are due to the same methods of attack, *i.e.*, through the study of particular diseases and by laboratory studies in nutrition.

It must not be supposed that chemical methods are not available to help establish the existence of vitamins for which evidence is first obtained in nutrition studies. The known properties of the established vitamins make it possible in many cases to find support for the existence of new vitamins by a process of elimination or by supplying the known vitamins in pure form, as is now done. Of special importance is the determination of the character of the chemical reactions or kind of processing procedures which destroy the new factors being sought. Such information is of great practical value to those engaged in the manufacture of various food products.

As already mentioned, one of the fascinating features of vitamin nutrition is the tremendous disproportion between their importance and the amounts normally consumed. Because they contribute no significant material substance or energy to the body, but act rather as catalysts of life processes it is necessary to place vitamins in an entirely different category from the classification of the better-known foodstuffs, such as proteins, fats, carbohydrates and mineral salts.

Vitamins are primarily plant products. There are certain exceptions to this rule. Ergosterol and other sterols, whether in plant or animal tissue, are transformed into vitamin D under the influence of the proper light rays and other forms of energy. Vitamin A is synthesized in animals from certain carotenoids which are formed only by plants. Another exception is that some species of animals synthesize vitamin C. It has been established for the rat,[2] the calf,[3] and the chicken,[4] that the liver contains an abundance of vitamin C when the animals have been fed for long periods or reared on diets which lack this factor. These findings show that these species are not dependent on their food for vitamin C, and show vitamin C synthesis in the animal itself. The lack of dependence of growing cattle on their rations

[2] Parsons, H. T., The Antiscorbutic Content of Certain Body Tissues of the Rat. The Persistence of the Antiscorbutic Substance in the Liver of the Rat after Long Intervals on a Scorbutic Diet, *J. Biol. Chem.*, 44: 587–602 (1920).

[3] Thurston, L. M., Eckles, C. H., and Palmer, L. S., The Role of the Antiscorbutic Vitamin in the Nutrition of Calves, *J. Dairy Sci.*, 9: 37–49 (1926); and Thurston, L. M., Palmer, L. S., and Eckles, C. H., Further Studies of the Role of Vitamin C in the Nutrition of Calves, *ibid.*, 12: 394–404 (1929).

[4] Carrick, C. W., and Hauge, S. M., Presence of the Antiscorbutic Substance in the Livers of Chickens Fed on Scorbutic Diets, *J. Biol. Chem.*, 63: 115–122 (1925).

for vitamin B (B_1)[5] has been explained by the finding[6] that this factor is synthesized by the bacteria inhabiting the rumen of this species. The human organism, however, apparently requires many of the vitamins at present recognized, although this has not been definitely established for all the vitamins mentioned at the beginning of this chapter. In addition, man is dependent on food for his vitamins, with the exception of vitamin D, which is formed in the epidermis after exposure to ultraviolet rays.

An important nutrition fact in connection with vitamins is the ability of animals to store these factors in the organs of the body, particularly in the liver. This is particularly true of vitamin A, and for fish, of vitamin D. In spite of the fact that the other vitamins are not stored in any great quantities, there is no evidence to indicate that excessive doses of the vitamins may occur through consumption of food.

The phases of vitamin knowledge which are probably of most interest from the biochemical standpoint have to do with the effects of the presence or absence of vitamins on animal life, their origin and distribution in nature, and their chemical properties. The subsequent paragraphs will present briefly such facts, bearing on these phases of the vitamins, as appear to be of special importance.

VITAMIN A.—Vitamin A was discovered almost simultaneously by McCollum and Davis[7] and by Osborne and Mendel.[8] The discovery was based on the observations, which have been repeatedly confirmed, that certain fats and oils possess a remarkable power of stimulating the growth of rats, which stimulatory effect is absent in whole or in part from other fats and which is not explainable on the basis of the glycerides contained in these fats. Several years later Drummond[9] studied in detail the known components of growth-promoting fats and was unable to identify the vitamin with glycerol, saturated or unsaturated fatty acids, cholesterol, lecithin, and other phospholipids.

Vitamin A is classified among the growth-promoting factors. It is not clear just how this effect is brought about. There is no evidence as yet that this or any other vitamin actually accelerates the metabolic processes or modifies their efficiency. The probabilities are that the growth-promoting effect of vitamin A is exerted indirectly through its

[5] Bechdel, S. I., Eckles, C. H., and Palmer, L. S., The Vitamin B Requirement of the Calf, *J. Dairy Sci.*, 9: 409–438 (1926).

[6] Bechdel, S. I., Honeywell, H. E., Dutcher, R. A., and Knutsen, M. H., Synthesis of Vitamin B in the Rumen of the Cow, *J. Biol. Chem.*, 80: 231–238 (1928).

[7] McCollum, E. V., and Davis, M., The Necessity of Certain Lipins in the Diet During Growth, *J. Biol. Chem.*, 15: 167–175 (1913).

[8] Osborne, T. B., and Mendel, L. B., The Relation of Growth to the Chemical Constituents of the Diet, *J. Biol. Chem.*, 15: 311–326 (1913).

[9] Drummond, J. C., Researches on the Fat-Soluble Accessory Substance. I. Observations upon Its Nature and Properties, *Biochem. J.*, 13: 81–94 (1919).

apparent ability to maintain the integrity of the epithelial tissues of the animal body. This is supported by the fact that the primary effect of a deficiency of vitamin A is a keratinization of the epithelial tissues. There is no specific disease which is characteristic of vitamin A deficiency. There is no longer any foundation for the general assumption that xerophthalmia is a specific disease due to lack of vitamin A. As a matter of fact, the diseased condition of the cornea, which is called xerophthalmia, is the result of bacterial invasion of the keratinized tissue.[10] Mori found the keratinized condition of the epithelium to extend to the salivary gland, mouth, larynx, trachea, bronchi, and lungs. These findings were verified by Wolbach and Howe,[11] who found in addition that the keratinization extended throughout the alimentary tract, the genital-urinary tract, and various ductless glands. These findings show why vitamin A deficiency in animals lowers their resistance to respiratory diseases and also explain the relation of the vitamin to reproduction. These facts emphasize the importance of this vitamin for human nutrition, particularly since it does not have a wide distribution in nature among the foods commonly employed by man.

Vitamin A is synthesized by animals only. The high vitamin A activity of green leaves and the apparent association of vitamin A potency with green and yellow seeds, and various fruits and roots with yellow carotenoid pigments,[12] is due to the fact that one or more of the carotenoids are the precursors from which vitamin A is formed.[13]

[10] Mori, S., Primary Changes in Eyes of Rats Which Result from Deficiency of Fat-Soluble A in Diet, *J. Am. Med. Assoc.*, 79: 197–200 (1922); The Changes in the Para-Ocular Glands Which Follow the Administration of Diets Low in Fat-Soluble A; With Notes of the Effect of the Same Diets on the Salivary Glands and the Mucosa of the Larynx and Trachea, *Bull. Johns Hopkins Hosp.*, 33: 357–359 (1922); and The Pathological Anatomy of Ophthalmia Produced by Diets Containing Fat-Soluble A, but Unfavorable Contents of Certain Inorganic Elements, *Am. J. Hyg.*, 3: 99–102 (1923).

[11] Wolbach, S. B., and Howe, P. R., Tissue Changes Following Deprivation of Fat-Soluble A Vitamin, *J. Exptl. Med.*, 42: 753–777 (1925).

[12] Steenbock, H., Boutwell, P. W., and Kent, H. E., Fat-Soluble Vitamine, I., *J. Biol. Chem.*, 35: 517–526 (1918); Steenbock, H., and Gross, E. G., II. The Fat-Soluble Vitamine Content of Roots, Together with Some Observations on Their Water-Soluble Vitamine Content, *ibid.*, 40: 501–531 (1919); Steenbock, H., and Boutwell, P. W., III. The Comparative Nutritive Value of White and Yellow Maizes, *ibid.*, 41: 81–96 (1920); Steenbock, H., and Gross, E. G., IV. The Fat-Soluble Vitamine Content of Green Plant Tissues Together with Some Observations on Their Water-Soluble Vitamine Content, *ibid.*, 41: 149–162 (1920); Steenbock, H., and Boutwell, P. W., VI. The Extractability of the Fat-Soluble Vitamine from Carrots, Alfalfa, and Yellow Corn by Fat Solvents, *ibid.*, 42: 131–152 (1920); and Steenbock, H., Sell, M. T., and Boutwell, P. W., VIII. The Fat-Soluble Vitamine Content of Peas in Relation to Their Pigmentation, *ibid.*, 47: 303–308 (1921).

[13] Moore, T., A Note on Carotin and Vitamin A, *The Lancet*, March 9, 1929,

This explains why the genetic factors for carotenoid pigmentation and vitamin A activity in yellow maize cannot be separated by crossing yellow and white varieties.[14] The conversion of carotenoids to vitamin A apparently occurs in the liver of animals under the influence of a hydrolytic enzyme, which has been called carotenase.[15] It may not be present in all higher animals.

Among the foods of animal origin which are usually reliable sources of vitamin A are whole milk, butter, cheese, egg yolk, and the glandular organs of animals, particularly the liver. The presence of vitamin A in these foods, however, depends upon the presence of carotenes and cryptoxanthin in the food of the animals which produce these products.[16] These products may contain both true vitamin A and one or more of its carotenoid precursors, usually chiefly β-carotene, although in egg yolk it is chiefly cryptoxanthin. Standard U.S.P. cod-liver oil is a rich source of vitamin A, but halibut- and burbot-liver oil are many times richer. This fact is of considerable importance when therapeutic doses of the vitamin are necessary. These high concentrations of vitamin A in the liver of fish represent the effect of diets exceptionally rich in carotenes during the fattening season. A similar relationship exists between the diet of cows and hens and the vitamin A content of milk and eggs. The highest concentrations of vitamin A in these foods occur during the season when an abundance of fresh green forage is available. Fortunately the lack of green forage for the winter feeding of dairy cattle and laying fowls may be overcome in large part through feeding properly cured, leafy forage, yellow seeds, such as yellow corn, and yellow roots. These facts are valuable to the producers of eggs and milk, in helping them control the vitamin A content of their respective products.

The experiments on vitamin A activity in plants[17] which were formerly regarded as indicating the physiological conditions under which plants produce this vitamin are now to be interpreted as im-

p. 499; The Relation of Carotin to Vitamin A, *ibid.*, August 24, 1929, p. 380; and Vitamin A and Carotine, I-VII, *Biochem. J.*, 23: 803–811, 1267–1272 (1929); 24: 672–712 (1930); 25: 275–286 (1931).

[14] Hauge, S. M., and Trost, J. F., An Inheritance Study of the Distribution of Vitamin A in Maize, *J. Biol. Chem.*, 80: 107–114 (1928).

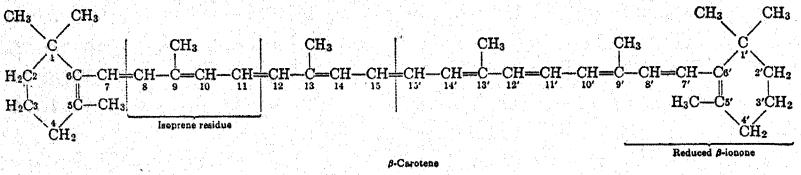
[15] Olcott, H. S., and McCann, D. C., The Transformation of Carotene to Vitamin A *in vitro*, *Science*, 74: 414 (1931); Carotenase. The Transformation of Carotene to Vitamin A *in vitro*, *J. Biol. Chem.*, 94: 185–193 (1931).

[16] Kennedy, C., and Dutcher, R. A., Vitamine Studies. IX. The Influence of the Diet of the Cow upon the Quantity of Vitamines A and B in the Milk, *J. Biol. Chem.*, 50: 339–359 (1922).

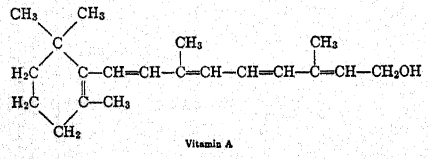
[17] Coward, K. H., The Influence of Light and Heat on the Formation of Vitamin A in Plant Tissues, *J. Biol. Chem.*, 72: 781–799 (1927); *cf.* also Moore, T., Vitamin A Formation in the Etiolated Wheat Shoot, *Biochem. J.*, 21: 870–874 (1927); and Heller, V. G., Vitamin Synthesis in Plants as Affected by Light Source, *J. Biol. Chem.*, 76: 499–511 (1928).

portant contributions to our knowledge regarding the synthesis of those carotenoids from which vitamin A may be formed in animal metabolism.

The chemical nature of vitamin A was established by the brilliant researches of Karrer [18] and associates, later confirmed by Heilbron, Morton, and Webster,[19] and by Karrer [20] himself by means of chemical synthesis. In fact, when Moore (*loc. cit.*) established the biochemical basis for vitamin A synthesis in the animal body from the plant pigment, carotene, the chemical basis for such a relationship had already been laid by the Karrer school. They had shown β-carotene to be an aliphatic methylated hydrocarbon having two identical, unsaturated, optically inactive terminal rings having the same structure as that occurring in β-ionone. Moreover, the aliphatic polyene central chain could be considered as a condensed chain of two pairs of dehydrated isoprene (β-methylbutadiene) residues united in reverse order at the center of the chain (carbons 15, 15′ in the structural formula). The carotene structure is evidently capable of hydrolysis at this point giving rise to two identical half (semi-) β-carotene alcohols (semi-β-carotenol). Partly on the basis of this deduction supported by much chemical evidence, including the demonstration of the β-ionone structure in highly purified vitamin A from fish-liver oil, Karrer proposed the structure for vitamin A which is now accepted. Heilbron, Morton,



β-Carotene



Vitamin A

and Webster substantiated Karrer's formula for vitamin A by forming from it 1-6-dimethylnaphthalene, which could be accounted for only

[18] Karrer, P., Helfenstein, A., Wehrli, H., and Wettstein, A., Pflanzenfarbstoffe. XXV. Über die Konstitution des Lycopins und Carotins, *Helv. Chim. Acta*, 13: 1084–1099 (1930); and Karrer, P., Morf, R., and Schöpp, K., Zur Kenntnis des Vitamins-A aus Fischtranen II, *ibid.*, 14: 1431–1436 (1931).

[19] Heilbron, I. M., Morton, R. A., and Webster, E. T., The Structure of Vitamin A, *Biochem. J.*, 26: 1194–1196 (1932).

[20] Karrer, P., Morf, R., and Schöpp, K., Synthese des Perhydrovitamins A, *Helv. Chim. Acta*, 16: 557–561 (1933).

by assuming the correctness of Karrer's formula. Karrer's school completed the proof by an eight-step synthesis of crystalline perhydrovitamin A, beginning with β-ionone and proving its identity with the completely hydrogenated natural vitamin from fish-liver oil. The vitamin itself has now been successfully synthesized,[21, 22] apparently in the one case through reduction of the aldehyde condensation product of β-cyclocitrol with two moles of dimethyl acrolein and in the other case through reduction of the same product produced by condensation of β-ionylideneacetaldehyde with β-methylcrotonaldehyde. The natural vitamin has also been obtained in crystalline form.[23]

The ring structure, the unsaturated polyene chain, and the aliphatic primary alcohol group, all are of major importance in the biochemistry of vitamin A. The unsaturated, unoxidized, optically inactive ring evidently plays some, as yet undetermined, role in the biological activity of the vitamin because only those carotenoids having the ionone ring which possesses these characteristics are convertible to vitamin A. Thus one molecule of β-carotene which has two such rings may form two molecules of vitamin A, whereas α-carotene, γ-carotene, and cryptoxanthin, the only other known carotenoids that may be converted to vitamin A, can yield only one molecule of the vitamin because they have only one such ring per molecule. Therefore the ring structure of vitamin A very definitely limits its carotenoid precursors. β-Carotene is by far the most important and widely distributed of the vitamin A precursors.

A certain amount of unsaturation in the vitamin A aliphatic chain evidently is required for biological activity because both perhydrovitamin A and perhydro-β-carotene are inactive while α- and β-dihydrocarotene and diiodocarotene are active. The side-chain unsaturation explains for the most part the probable function of vitamin A and the carotenoids as oxidation reduction catalysts; they should be both hydrogen acceptors and oxidation promoters, the latter especially when actively absorbing oxygen, as they readily do in solution (in fat and fat solvents). Highly oxidized carotenes and vitamin A are not biologically active when oxidized, an important fact for vitamin A preservation in foods.

The alcohol structure in vitamin A explains its ability to form compounds with fat acids, proteins, bile acids, etc., all of which are evidently important in its biochemistry, namely, the absorption of the

[21] Fuson, R. C., and Christ, R. E., The Condensation of β-Cyclocitrol with Dimethylacrolein, *Science*, 84: 294–295 (1936).

[22] Kuhn, R., and Morris, C. J. O. R., Synthese des Vitamins A, *Ber.*, 70B: 853–858 (1937).

[23] Holmes, H. N., and Corbet, Ruth E., A Crystalline Vitamin A Concentrate, *Science*, 85: 103 (1937); and The Isolation of Crystalline Vitamin A, *J. Am. Chem. Soc.*, 59: 2042–2047 (1937).

vitamin from the intestine as bile acid compound,[24] its transportation in the blood and lymph as fat acid ester,[25] its storage in the liver [26] as similar compound, the apparent occurrence of protein-vitamin A [27] compound as precursor of the visual purple of the retina, and the appearance of free vitamin A in non-saponifiable extracts from vitamin A-containing oils and fats. However, the vitamin, being an alcohol, may be separated from its carotenoid precursors by the well-known phase-separation procedure so widely employed in carotenoid chemistry. Vitamin A accompanies the "xanthophylls" in the alcohol layer of a petroleum ether-80–90 per cent methyl alcohol separation.

Vitamin A and each of its carotenoid precursors exhibit characteristic spectroscopic absorption bands, the most important bands of the carotenoids being in the visible blue and violet, that of vitamin A being in the invisible ultraviolet. The wave length of maximum intensity, 328 mμ, of the vitamin A absorption band (300 mμ–350 mμ) has proved to be of great importance for the quantitative spectrophotometric [28] or photoelectric [29] estimation of the vitamin. Although not specific for vitamin A, since dehydroergosterol also gives a maximum at 328 mμ, it can be identified because the sterols show three other maxima in the ultraviolet. By using a wave length of maximum intensity of one of the visible bands of the carotenoid precursors of vitamin A, they too can be estimated by spectrophotometric methods, since the absorption intensity depends upon the molar concentration of the pigment layer at the point chosen. For detailed description of theory and methods, see Zscheile, Hogness, and Young,[30] and Miller.[31]

[24] Greaves, J. D., and Schmidt, C. L. A., The Utilization of Carotene by Jaundiced and Phosphorus-treated Vitamin A-deficient Rats, *Am. J. Physiol.*, 111: 502–506 (1935).

[25] Drummond, J. C., Bell, M. E., and Palmer, E. T., Observations on the Absorption of Carotene and Vitamin A, *Brit. Med. J.* (1935), 1: 1208–1210.

[26] Reti, L., Sur l'état de combinaison de la vitamine A dans les huiles de foie, *Compt. rend. soc. biol.*, 120: 577–580 (1935).

[27] Wald, George, Carotenoids and the Visual Cycle, *J. Gen. Physiol.*, 19: 351–371 (1935).

[28] Holmes, A. D., Black, A., Eckler, C. R., Emmett, A. D., Heyl, F. W., Neilsen, C., and Quinn, E. J., The Practical Application of the Spectrophotometric Method for Assay of Vitamin A, *J. Am. Pharm. Assoc.*, 26: 525–540 (1937).

[29] McFarlan, R. L., Reddie, J. W., and Merrill, E. C., A New Photoelectric Method for Measuring Vitamin A, *Ind. Eng. Chem.* (*Anal. Ed.*), 9: 324–326 (1937).

[30] Zscheile, F. P., Jr., Hogness, T. R., and Young, T. F., The Precision and Accuracy of a Photoelectric Method for Comparison of the Low Light Intensities Involved in Measurement of Absorption and Fluorescence Spectra, *J. Phys. Chem.*, 38: 1–11 (1934).

[31] Miller, E. S., The Application of Quantitative Spectral Analyses to Binary Mixtures of the Common Carotenoids, *Plant Physiol.*, 9: 681–684 (1934); A Rapid and Accurate Quantitative Method for the Determination of the Common Carotenoids, *J. Am. Chem. Soc.*, 57: 347–349 (1935); Improved Methods for the

The carotenes form an intensely blue compound with $SbCl_3$, and vitamin A a blue-violet compound with this salt under proper conditions, which have been studied extensively and applied both to the detection of vitamin A and its carotenoid precursors in animal tissues and products and to a quantitative colorimetric method, first developed by Carr and Price.[32] It is useful for preliminary assays of cod-liver oil and other vitamin A-rich fish-liver oils and for exploratory research in the physiology of the vitamin. The reaction with $SbCl_3$, however, is a general one for polyene substances.

For biological purposes vitamin A and provitamin concentrations in natural foods and medicinal products are not expressed in the usual quantitative chemical terms but in terms of units equivalent to the units of a standard product. By agreement the international standard for vitamin A is a sample of pure, crystalline β-carotene, a unit of which is the vitamin A activity of 0.6 μg. of the International Standard Preparation. Translated into medicinal products and natural foods, U.S.P. cod-liver oil must contain at least 600 units of vitamin per gram or something over 17,000 units per fluid ounce, while the best summer butter (richest in vitamin A) may average 1,150 units per ounce (18,000 units per pound) and the poorest winter butter (lowest in vitamin A) may contain only 150 units per ounce (2,500 units per pound). The carotene in carrots may be equivalent to 1,000 to 2,000 units per ounce and that in spinach from 2,500 to 10,000 units per ounce.

From the data and considerations of Guilbert, Miller, and Hughes,[33] and of others,[34] it is probable that the minimum vitamin A requirements of farm mammals (and rats) is 10–12 units per day per kilogram body weight, administered as vitamin A, and about 40 units administered as carotene, while a more optimum requirement for humans is 35–60 units per day per kilogram body weight for children from infancy to adolescence, the higher requirement being for infants, adults needing 17–20 units daily per kilogram body weight. Such important questions, however, cannot be said to have been completely settled.

THIAMIN CHLORIDE.—This is the name suggested by Williams[35] for the antineuritic or antiberiberi vitamin commonly called B or B_1.

Purification of the Common Carotenoids, and the Quantitative Measurement of their Absorption Spectra, *Botan. Gaz.*, 96: 447–467 (1935).

[32] Carr, F. H., and Price, E. A., Colour Reactions Attributed to Vitamin A, *Biochem. J.*, 20: 497–501 (1926). A special tintometer and a vitameter-A (by Hilger) are designed for quantitative work, utilizing this reaction.

[33] Guilbert, H. R., Miller, R. F., and Hughes, E. H., The Minimum Vitamin A and Carotene Requirement of Cattle, Sheep and Swine, *J. Nutrition*, 13: 543–564 (1937).

[34] *Cf.* Eddy, W. H., and Dalldorf, G., The Avitaminoses, pp. 6, 18–21, The Williams and Wilkins Company, Baltimore (1937).

[35] *Cf. J. Am. Med. Assoc.*, 109: 952 (1937).

The term aneurin, suggested by Jansen,[36] is also employed. Torulin and oryzanin, names employed first for crude preparations of the vitamin, are used by some authors for the pure compound also.

So many workers contributed to the early research on experimental and natural beriberi that it is not possible to credit the discovery of the antiberiberi vitamin to one person. Eijkman [37] is credited with first producing beriberi experimentally, and his experiments may now be interpreted as demonstrating the existence of an antiberiberi food factor, although he did not so interpret it until later.[38] Grijns [39] first suggested a nutritive deficiency theory for experimental polyneuritis and beriberi and made some attempts to determine the nature of the nutritive substance. The first claim of isolation of antiberiberi substance from foods was made by Hulshoff-Pol,[40] who called it X-acid. An intensive search for the substance during the years 1910–1912 reached a milestone in the history of vitamins with the coining of the term "vitamine" by Funk [41] for the substance which he believed to be the specific antineuritic substance. However, it was not until fifteen years later that the actual isolation of the pure crystalline vitamin was accomplished by Jansen and Donath,[42] although highly concentrated preparations had already been made by Seidell,[43] and others. Rapid progress was now possible in determining the chemistry of the vitamin. The presence of sulfur in the molecule was discovered by Windaus [44] and associates and confirmed by van Veen.[45] Methods

[36] Jansen, B. C. P., Identity of Vitamin B_2 and Flavine and the Nomenclature of Vitamins, *Nature*, 135: 267 (1935).

[37] Eijkman, C., Polyneuritis bij Hoenderen, *Geneesk. Tijdschr. v. Ned.-Ind.*, 30: 295–334 (1890); Polyneuritis bij Hoenders, Nieuwe Bijdrage tot de Aetiologie der Ziekte, *Geneesk. Tijdschr. v. Ned-Ind.*, 36: 214–269 (1896).

[38] Eijkman, C., Über Ernährungspolyneuritis, *Arch. Hyg.*, 58: 150–170 (1906).

[39] Grijns, G., Over polyneuritis gallinarum, *Geneesk. Tijdschr. v. Ned.-Ind.*, 41: 3–111 (1901); II. *ibid.*, 49: 216–238 (1909); III. *Mededeelingen Geneesk. Lab.*, 1908 and 1909, 31 pp. IV. *Geneesk. Tijdschr. v. Ned.-Ind.*, 51: 591–610 (1911). These papers have been published in English, *i.e.*, Professor Dr. G. Grijns' Researches on Vitamins, 1900–1911, J. Noorduyn and Son, Gorinchem, Holland (1935).

[40] Hulshoff-Pol, D. J., X-zuur; het tegen beri-beri verkzame bestanddeel uit de Katjang-hidjoe, *Geneesk. Tijdschr. v. Ned.-Ind.*, 47: 688–702 (1907).

[41] Funk, C., The Etiology of the Deficiency Diseases, *J. State Med.*, 20: 341–368 (1912).

[42] Jansen, B. C. P., and Donath, W. F., Isolation of Anti-beriberi Vitamin, *Geneesk. Tijdschr. v. Ned.-Ind.*, 66: 810–827 (1927).

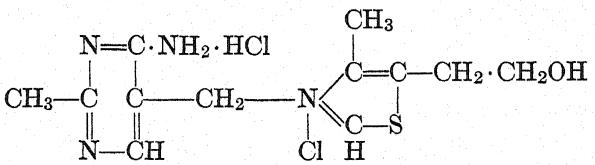
[43] Seidell, A., Further Experiments on the Isolation of the Antineuritic Vitamin, *J. Am. Chem. Soc.*, 44: 2042–2051 (1922); The Chemistry of Vitamins, *Science*, 60: 439–447 (1924).

[44] Windaus, A., Tschesche, R., Ruhkopf, H., Laquer, F., and Schultz, F., Die Darstellung von krystallisiertem antineuritischem Vitamin aus Hefe, *Z. physiol. Chem.*, 204: 123–128 (1932).

[45] van Veen, A. G., Die aus Hefe und Reiskleie gewonnenen krystallinischen Präparate des Vitamins B, *Z. physiol. Chem.*, 208: 125–128 (1932).

for obtaining larger yields of pure vitamin were devised by Williams [46] and associates and by Kinnersley, O'Brien, and Peters,[47] which made possible the practical employment, for the first time, of the pure antineuritic antiberiberi substance, as well as the determination of its chemical structure. Windaus, Tschesche, and Grewe [48] and Williams [49] demonstrated that the vitamin contains a pyrimidine and a thiazole ring. Makino and Imai [50] presented a number of convincing arguments for the existence of a saturated carbon atom uniting the two cyclic groups. This was confirmed by Williams,[51] who not only gave the first correct structural formula for the vitamin but soon followed [52] with a description of its synthesis, thus bringing to a successful conclusion the studies on the chemical nature of the first vitamin to be definitely postulated. The synthesis of the vitamin, presumably by other procedures, was also accomplished in 1936 by Andersag and Westphal, according to Grewe.[53]

Thiamin chloride hydrochloride has the following structure:



It is a colorless, crystalline compound melting at 232–234° C. Below *p*H 7 its aqueous solution shows two characteristic absorption maxima in the ultraviolet at 235 mμ and 267 mμ; its two acid-binding groups have *p*K values of 3.4 and 4.8, the former being that of the thiazole

[46] Williams, R. R., Waterman, R. E., and Keresztesy, J. C., Larger Yields of Crystalline Antineuritic Vitamin, *J. Am. Chem. Soc.*, 56: 1187–1191 (1934).

[47] Kinnersley, H. W., O'Brien, J. R., and Peters, R. A., Crystalline Vitamin B_1, *Biochem. J.*, 29: 701–715 (1935).

[48] Windaus, A., Tschesche, R., and Grewe, R., Über das antineuritische Vitamin, *Z. physiol. Chem.*, 237: 98–104 (1935).

[49] Williams, R. R., Structure of Vitamin B, *J. Am. Chem. Soc.*, 57: 229–230 (1935).

[50] Makino, K., and Imai, T., Bermerkung über die Chemie des antineuritischen Vitamins, *Z. physiol. Chem.*, 239: I–II (1936).

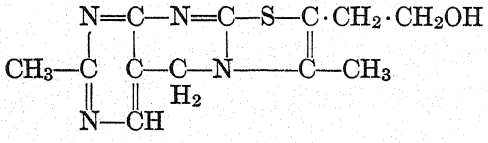
[51] Williams, R. R., Structure of Vitamin B_1, *J. Am. Chem. Soc.*, 58: 1063–1064 (1936).

[52] Williams, R. R., and Cline, J. K., Synthesis of Vitamin B_1, *J. Am. Chem. Soc.*, 58: 1504–1505 (1936); Cline, J. K., Williams, R. R., and Finkelstein, J., Studies of Crystalline Vitamin B_1, *J. Am. Chem. Soc.*, 59: 1052–1054 (1937); *cf.* also Williams, R. R., The Beriberi Vitamin, *Ind. Eng. Chem.*, 29: 980–984 (1937); The Chemistry of Thiamin (Vitamin B_1), *J. Am. Med. Assoc.*, 110: 727–732 (1938).

[53] Grewe, R., Über das antineuritische Vitamin. V., *Z. physiol. Chem.*, 242: 89–96 (1936).

nitrogen, the latter of the free amino group on position-6 of the pyrimidine half of the molecule. When a formaldehyde-treated solution of the compound is added to an alkaline diazotized sulfanilic acid, a characteristic yellow → pink color reaction is shown. This formaldehyde-azo reaction, first described by Kinnersley and Peters,[54] is stable when applied to thiamin chloride solutions which have not been treated with alkali above *p*H 7. It is a reaction given by the thiazole half of the molecule. Strong alkali causes the liberation of H_2S (especially when heated), the disappearance of the azo-color reaction, the appearance of a pseudo-acid group with *p*K about 9 (probably the alcohol group), and a marked change in the spectroscopic absorption maximum at 234 mμ, this showing first an increase in intensity and then being replaced by a broad, rapidly fading band at 330–340 mμ. Biological activity is gradually lost on heating in alkaline medium above *p*H 11.

One of the most interesting properties of thiamin is its conversion on oxidation under suitable conditions to a yellow-blue fluorescent pigment called thiochrome by Kuhn, Wagner-Jauregg, *et al.*[55] This substance has the following structure, showing that its formation from the oxidized vitamin involves loss of water as well as dehydrogenation and the consequent ring formation and shift of double bond. The



identity of thiochrome from yeast and the alkaline oxidation products of thiamin was proved by the researches of Kuhn and Vetter,[56] Barger, Bergel, and Todd,[57] and finally by Todd, Bergel, and Karimullah [58] and Bergel and Todd,[59] who have synthesized the pigment. Indeed, the synthesis of thiochrome furnished independent proof of the correct structure of the vitamin.

[54] Kinnersley, H. W., and Peters, R. A., The Formaldehyde-azo-test for Vitamin B_1, *Biochem. J.*, 28: 667–670 (1934).

[55] Kuhn, R., Wagner-Jauregg, T., van Klaveren, F. W., and Vetter, H., Über einen gelben, schwefelhaltigen Farbstoff aus Hefe, *Z. physiol. Chem.*, 234: 196–200 (1935).

[56] Kuhn, R., and Vetter, H., Zur Kenntnis des Thiochroms, *Ber.*, 68B: 2375–2385 (1935).

[57] Barger, G., Bergel, F., and Todd, A. R., Über das Thiochrom als Vitamin B_1 (Antineurin), *Ber.*, 68B: 2257–2262 (1935).

[58] Todd, A. R., Bergel, F., and Karimullah, Über Aneurin II. Über die Synthese von N-Aryl-thiazoliumsalzen; Über Einzelheiten in der Konstitution des Aneurins und Thiochroms, *Ber.*, 69B: 217–223 (1936).

[59] Bergel, F., and Todd, A. R., Synthesis of Thiochrome, *Nature*, 138:406 (1936).

Thiochrome itself is not biologically active, although Kinnersley, O'Brien, and Peters,[60] who suggest that the pigment belongs to a class of substances which they have named quinochromes, believe that the initial fluorescent oxidation products leading to the yellow pigment are biologically active and may occur in natural products as a form of the vitamin. A chemical method for the estimation of the vitamin, based on the quantitative formation of the fluorescent pigment from the vitamin, has been devised by Jansen.[61] Its application to natural products has presented difficulties in the isolation and preparation of sufficiently pure extracts.

It has long been a popular theory that thiamin chloride is concerned with the metabolism of carbohydrates. In fact, the only specific metabolic function of the vitamin for which there is definite biochemical evidence is its catalytic effect on the removal of pyruvic acid which accumulates in the brain in the acute head-retraction stage of experimental beriberi. This was shown first by Meiklejohn, Passmore, and Peters [62] and led to the catatorulin test, an *in vitro* test on polyneuritic pigeon brain tissue devised by Passmore, Peters, and Sinclair [63] and used so extensively by the Oxford University biochemists and others in studies of the antineuritic vitamin. Mathews' [64] suggestion that the vitamin may act as a respiratory catalyst by union with phosphoric acid and a suitable protein has been confirmed by Lohmann and Schuster,[65] who have isolated from yeast a pyrophosphoric acid ester of thiamin chloride which is a specific coenzyme for the carboxylase in the oxidation of pyruvic acid. Tauber [65a] has shown that the synthesis of the pyrophosphoric acid ester takes place in the intestinal mucosa. According to Lipmann [65b] animal tissue contains

[60] Kinnersley, H. W., O'Brien, J. R., and Peters, R. A., The Properties of Blue Fluorescent Substances Formed by Oxidation of Vitamin B_1 (Quinochromes), *Biochem. J.*, 29: 2369–2384 (1935).

[61] Jansen, B. C. P., A Chemical Determination of Aneurin (= vitamin B_1) by the Thiochrome Reaction, *Rec. trav. chim.*, 55: 1046–1052 (1936).

[62] Meiklejohn, A. P., Passmore, R., and Peters, R. A., Pyruvic Acid and Vitamin B_1 Deficiency, *Biochem. J.*, 26: 1872–1879 (1932).

[63] Passmore, R., Peters, R. A., and Sinclair, H. M., Catatorulin. A New Method for Comparing the Oxidative Factor in Vitamin B_1 Concentrates, *Biochem. J.*, 27: 842–850 (1933); *cf.* also Kinnersley, H. W., O'Brien, J. R., and Peters, R. A., *Biochem. J.*, 29: 713 (1935) for modification of the method.

[64] Mathews, A. P., Principles of Biochemistry, p. 425, William Wood and Company, Baltimore (1936).

[65] Lohmann, K., and Schuster, Ph., Co-carboxylase, *Naturwissenschaften*, 25: 26–27 (1937); *Z. angew. Chem.*, 50: 221 (1937).

[65a] Tauber, H., Enzymic Synthesis of Cocarboxylase from Vitamin B_1. *Enzymologia*, 2: 171–174 (1937).

[65b] Lipmann, F., Die Dehydrierung der Brenztraubensäure, *Enzymologia*, 4: 65–72 (1937).

thiamin solely in the ester form, but Tauber [65c] finds that in plant tissue only a small part of the vitamin is present as the ester.

Whether the carbohydrate metabolism in tissues other than those of the nervous system is affected by thiamin chloride deficiency is not so certain. Thompson [66] found that the oxygen up-take of the kidney was affected but not that of muscle, heart, and liver tissue. Later [67] he reported abnormally large amounts of pyruvate in the blood of thiamin chloride deficient pigeons and rats, but this was not confirmed by de Jong.[68] However, support for the idea of a general relationship between this vitamin and carbohydrate metabolism is found in the series of biological experiments of Evans and Lepkovsky,[69] and Salmon and Goodman [70] on the sparing action of dietary fat on the nutritive requirements for the vitamin. Salmon and Goodman found evidence of a relationship between the length of the carbon chain of fatty acids and their effectiveness; the maximum was for eight carbon acids, the effect decreasing with either less or greater length of the chain. McHenry [71] believes the vitamin catalyzes oxidation of pyruvate in nervous tissue and the synthesis of fat from pyruvate in other tissues, and is thus a specific agent in the intermediary metabolism of carbohydrate, the end product of which depends on the tissue involved.

Although the biochemical defect of thiamin chloride deficiency has been demonstrated only with the brain and kidney tissue, it seems probable that like effects occur also in the peripheral nerves and are responsible for some of the other well-known pathological effects of the vitamin deficiency such as hypomotility and atony of the gastrointestinal tract (which must be related to the specific anorexia of thiamin chloride deficiency), the decreased heart beat (bradycardia), and other changes for which such a defect in nerve metabolism could be responsible.

The initial biochemical defects in nerve tissue are followed in more advanced stages by serious anatomic lesions, which occur primarily in

[65c] Tauber, H., Co-Carboxylase (Vitamin B_1-Pyrophosphate) Content of Plants, *Proc. Soc. Exptl. Biol. Med.*, 37: 541–543 (1937).

[66] Thompson, R. H. S., The Action of Crystalline Vitamin B_1 on the Respiration of Polyneuritic Tissues *in Vitro*, *Biochem. J.*, 28: 909–915 (1934).

[67] Thompson, R. H. S., and Johnson, R. E., Blood Pyruvate in Vitamin B_1 Deficiency, *Biochem. J.*, 29: 694–700 (1935).

[68] de Jong, S., Bisulphite-binding Capacity of the Blood of Pigeons with B_1 Deficiency, *Arch. neérland. physiol.*, 21: 465–475 (1936).

[69] Evans, H. M., and Lepkovsky, S., and Murphy, E. A., The Sparing Action of Fat on Vitamin B. VII. The Effectiveness of Various Natural Fats in Sparing Vitamin B, *J. Biol. Chem.*, 107: 439–442 (1934). The previous papers in the series are cited here and in the paper by de Jong.

[70] Salmon, W. D., and Goodman, J. G., Alleviation of Vitamin B Deficiency in the Rat by Certain Fats and Synthetic Esters, *J. Nutrition*, 13: 477–500 (1937).

[71] McHenry, E. W., Vitamin B_1 and the Synthesis of Fat from Carbohydrate, *Science*, 86: 200 (1937).

the peripheral nerves, especially in the distal portions. For a detailed discussion of the pathology of various degrees of thiamin chloride deficiency and beriberi consult Eddy and Dalldorf.[72]

The thiamin chloride content of natural foods, special products rich in the vitamin, such as yeast, wheat germ, and rice polish, and of proprietary concentrates has been estimated in most instances by biological methods. Most commonly employed have been growth rate of rats, prevention or cure of experimental polyneuritis in pigeons, or cure of polyneuritis in rats. Recently the results of biological thiamin chloride assays of foods, etc., and even of the pure vitamin have been expressed in terms of International Units. This has been possible through a comparison of one or more defined effects of determined quantities of the unknown with the same effects of definite amount of International Standard, 10 mg. of which exhibit 1 Unit of activity, according to agreement. The Standard is a specific sample of fuller's earth containing adsorbed thiamin chloride from an extract of rice polish, the extract and adsorption product having been made under carefully defined conditions. Eventually the pure vitamin will, of course, be the International Standard. Jansen, employing the rat-growth method [73] and the thiochrome method,[74] estimated that 3 μg. of pure thiamin chloride hydrochloride exhibit 1 Unit of activity. According to the report of the Conference which adopted the standard and defined the unit, the quantity of vitamin required to maintain normal growth in a young rat is 1 to 2 Units daily, and the curative "daily dose" for acute pigeon polyneuritis is 1 to 3 Units. Kinnersley, O'Brien, and Peters,[75] using natural thiamin chloride hydrochloride from several sources and employing several methods of assay (biological, spectroscopic, and diazo color reaction), arrived at an average activity of 380–490 (average 437) International Units per milligram.[76] The manufacturers of "Betaxin" claim that 2 μg. of their synthetic product are equivalent to 1 Unit, or 500 Units per milligram, while the manufacturers of synthetic "Betabion" claim only 300 Units. Ammerman and Waterman,[77] using the natural thiamin chloride hy-

[72] Eddy, W. H., and Dalldorf, G., The Avitaminoses, Williams and Wilkins Company, Baltimore, Maryland (1937).

[73] Jansen, B. C. P., Wertbestimmung des Völkerbund-Standart-Präparats für Vitamin B_1, *Z. Vitaminforsch.*, 5: 254–256 (1936).

[74] Jansen, B. C. P., A Chemical Determination of Aneurin (=Vitamin B_1) by the Thiochrome Reaction, *Rec. trav. chim.*, 55: 1046–1052 (1936).

[75] Kinnersley, H. W., O'Brien, J. R., and Peters, R. A., Crystalline Vitamin B_1, *Biochem. J.*, 29: 701–715 (1935).

[76] Erroneously reported in article as 437 units per *gram*.

[77] Ammerman, M., and Waterman, R. E., Studies of Crystalline Vitamin B. IV. Injection Method of Assay, *J. Nutrition*, 10: 25–33 (1935); Waterman, R. E., and Ammerman, M., Studies of Crystalline Vitamin B. V. Effect of Graduated Doses on Growing Rats, *ibid.*, 10: 35–44 (1935); and VI. Effect of Graduated Doses on Pigeons, *ibid.*, 10: 161–166 (1935).

drochloride in an extended study, found increasing growth stimulation in rats from daily doses ranging from 0.5 μg. to 160 μg. and a minimum curative dose of 4 μg. for polyneuritic pigeons. The curative dose (lasting 5–6 days) for polyneuritic rats of the synthetic thiamin chloride hydrochloride is 6 μg. according to Cline, Williams, and Finkelstein.[78]

It is possible to conceive that microorganisms are the primary source of thiamin chloride in nature. Higher plants appear to synthesize it and possibly employ it in their metabolism; they concentrate it in the seeds, especially in the embryo. Animals as widely separated, biologically, as mammals and insects require the vitamin in their food or must live symbiotically with microorganisms capable of forming it. The role of bacteria in supplying the thiamin chloride requirements of ruminants was discovered by Bechdel *et al.*[79] The literature regarding insects and symbiotic microorganisms is reviewed by van't Hoog.[80]

Thiamin chloride is fairly abundant in animal products normally selected for the human dietary and withstands the usual cooking processes. Biological assays which have been made show 10 to 50 International Units per ounce in meat products, 5 to 20 Units per ounce in fish and shellfish, and 3 to 8 Units per ounce in whole milk and eggs, dried milk having 21 to 28 Units and egg yolk 13 to 40 Units per ounce in the assays reported. The vitamin is widely distributed in vegetables and cereals. Of about 40 vegetables listed by Eddy and Dalldorf (*loc. cit.*) nearly 30 had less than 10 Units per ounce, beans (kidney, lima, string), peas, lentils, and asparagus (green) being the richest of the vegetables in thiamin chloride, showing 20 to 45 Units per ounce. Of the cereals only the degerminated seeds and the flour made from them are poor sources of thiamin chloride. Values reported range from 20 to 50 Units per ounce for the whole cereals. Fruits as a whole are poor sources of thiamin chloride, most of them having considerably less than 10 Units per ounce. Nuts, however, are rich in the vitamin, especially peanuts, with 56 to 90 Units per ounce.

The thiamin chloride requirements of man and the domestic animals are reasonably well established in terms of minimum needs for protection against beriberi and polyneuritis. Cowgill[81] has established beyond reasonable doubt that the requirements for this vitamin

[78] Cline, J. K., Williams, R. R., and Finkelstein, J., Studies on Crystalline Vitamin B_1, *J. Am. Chem. Soc.*, 59: 1052–1054 (1937).

[79] Bechdel, S. I., Honeywell, H. E., Dutcher, R. A., and Knutsen, M. H., Synthesis of Vitamin B in the Rumen of the Cow, *J. Biol. Chem.*, 80: 231–238 (1928).

[80] van't Hoog, E. G., Aseptic Culture of Insects in Vitamin Research, *Z. Vitaminforsch.*, 4: 300–324 (1935); *ibid.*, 5: 118–126 (1936).

[81] Cowgill, G. R., The Vitamin B Requirement of Man, Yale University Press, New Haven, Connecticut (1934).

may be expressed in relation to the caloric value of the food consumed. This was developed from the thesis that "the vitamin requirement per unit of body mass is proportional to the metabolism of that mass." Using an arbitrary vitamin unit, called milligram-equivalent, Cowgill worked out a normal ratio of vitamin intake to Calorie intake for any body weight based on the formula

$$\frac{\text{Vitamin intake (Cowgill Units)}}{\text{Calorie intake}} = 0.0000284 \text{ Body weight (grams)}.$$

This calculated ratio is about 2.00 for a 70-kilo person and about 1.40 for a 50-kilo person. Using Cowgill's estimate that 1 International Unit equals 20 milligram-equivalents, this ratio gives a normal requirement of 250 International Units of thiamin chloride for a 70-kilo person, which is not far above the estimate of 200 International Units required daily to protect an adult against beriberi. In terms of pure thiamin hydrochloride this is either 0.4 to 0.5 mg. or 0.6 to 0.75 mg. daily depending on the correct μg. equivalents of 1 Unit and whether the requirement is 200 or 250 Units. Optimum requirements have been estimated as 500-600 Units daily.

l-Ascorbic Acid.—Ascorbic acid is the name given the antiscorbutic vitamin by Szent-Györgyi and Haworth.[82] It is also commonly called vitamin C. In the American medical literature it is called cevitamic acid. The substance normally crystallizes in rectangular plates which show straight extinction with high birefringence [$n\alpha$ (D line) 1.47 $\pm$ 0.005; $n\beta$ (D line) 1.68 $\pm$ 0.005]. The crystals melt at 192° C. (uncorrected) in open capillary. The specific rotation of the *l*-series compound (the biologically active form) is $[\alpha]_D^{20°} = +24°$ (1 per cent aqueous solution), without mutarotation. Aqueous solutions show one spectroscopic absorption maximum at 260 mμ. The molecular extinction coefficient for a solution containing 20 mg. per liter is 10,000 (aqueous solution containing 0.1 ml. 0.1 *N* H_2SO_4 per 100 ml.). Aqueous solutions exhibit two dissociation constants, pK′=4.21 and pK″=11.57, and an oxidation-reduction potential $E'_0 = +0.136$ volt at pH 4.58 and 30° C. Ten milligrams of *l*-ascorbic acid require 11.4 ml. 0.01 *N* aqueous iodine, the titration being carried out with starch indicator.

The classic studies of Holst,[83] Holst and Frölich,[84] and Fürst[85]

[82] Szent-Györgyi, A., and Haworth, W. N., "Hexuronic Acid" (Ascorbic Acid) as the Antiscorbutic Factor, *Nature*, 131: 24 (1933).

[83] Holst, A., Experimental Studies Relating to "Ship-Beri-Beri" and Scurvy, *J. Hyg.*, 7: 619–633 (1907).

[84] Holst, A., and Frölich, T., On the Etiology of Scurvy, *J. Hyg.*, 7: 634–671 (1907); and Über Experimentellen Skorbut, *Z. Hyg. Infektionskrankh.*, 72: 1–120 (1912).

[85] Fürst, V., Weitere Beiträge zur Ätiologie des Experimentellen Skorbuts des Meerschweinchens, *Z. Hyg. Infektionskrankh.*, 72: 121–154 (1912).

furnished the first experimental proof of the existence of an antiscorbutic vitamin, although they were conducted prior to the development of the vitamin hypothesis. However, certain difficulties of technic in producing experimental scurvy and failure to understand the proper relation of the other known vitamins, then called A and B, as well as lack of appreciation of the differences in susceptibility of different species of animals to scurvy, prevented the general acceptance of a vitamin-deficiency etiology of scurvy until about 1919.[86]

The instability of the antiscorbutic vitamin was early recognized as one of its most important properties, and was a serious obstacle to its isolation. The demonstration by numerous workers in 1921 that the vitamin is especially susceptible to oxidation, accelerated by alkalinity, was an important advance. The hypothesis of LaMer, Campbell, and Sherman [87] that heat destruction of the vitamin is an intramolecular oxidation-reduction has been substantiated. Serious attempts to concentrate the antiscorbutic vitamin began with Harden and Zilva.[88] Connell and Zilva [89] concluded from permeability studies that the size of the active vitamin molecule was of the order of a hexose, which has also been substantiated. The strong reducing properties of preparations of the vitamin were discovered by Zilva,[90] who later [91] pointed out both the alternate reduction-oxidation character of the reaction towards phenolindophenol and the quantitative reduction of the indicator by both decitrated lemon juice and active vitamin fractions. The latter property, which Zilva [92] unfortunately did not at first accept as a property of the vitamin but rather of some accompanying substance, was developed by Tillmans [93] and Tillmans and associates [94] as a method for the quantitative estimation of the

[86] Drummond, J. C., Note on the Role of the Antiscorbutic Factor in Nutrition, *Biochem. J.*, 13: 77–80 (1919).

[87] LaMer, V. K., Campbell, H. L., and Sherman, H. C., The Effect of Temperature and the Concentration of Hydrogen Ions upon the Rate of Destruction of Antiscorbutic Vitamin (Vitamin C), *J. Am. Chem. Soc.*, 44: 172–181 (1922).

[88] Harden, A., and Zilva, S. S., The Antiscorbutic Factor in Lemon Juice, *Biochem. J.*, 12: 259–269 (1918).

[89] Connell, S. J. B., and Zilva, S. S., The Differential Dialysis of the Antiscorbutic Factor, II., *Biochem. J.*, 18: 641–646 (1924).

[90] Zilva, S. S., The Antiscorbutic Fraction of Lemon Juice, II., *Biochem. J.*, 18: 632–637 (1924).

[91] Zilva, S. S., The Antiscorbutic Fraction of Lemon Juice, V., *Biochem. J.*, 21: 689–697 (1927).

[92] Zilva, S. S., The Non-Specificity of the Phenolindophenol Reducing Capacity of Lemon Juice and its Fractions as a Measure of their Antiscorbutic Activity, *Biochem. J.*, 26: 1624–1627 (1932).

[93] Tillmans, J., Das antiscorbutische Vitamin, *Z. Untersuch. Lebensm.*, 60: 34–44 (1930).

[94] Tillmans, J., Hirsch, P., and Hirsch, W., Das Reduktionsvermögen pflanzlicher Lebensmittel und seine Beziehung zum Vitamin C. 1. Der reduzierende

vitamin. Modifications of this method by Harris and associates,[95] by Bessey and King,[96] by Emmerie and van Eekelen [97] and by others are widely used in nutrition experiments and in the clinical detection of ascorbic acid deficiency, Harris and Ray [98] having demonstrated its value for this purpose. The attempts to isolate the antiscorbutic vitamin were continued for more than a decade, especially in Zilva's laboratory at the Lister Institute in London, by Bezssonoff at Strasburg, and later by King and associates at the University of Pittsburgh. King and Waugh [99] were the first to report the isolation of the pure crystalline vitamin and the demonstration of its biological activity in 0.5-mg. daily doses to guinea pigs. They recognized the apparent identity of the vitamin with the strongly reducing substance previously isolated by Szent-Györgyi [100] from adrenal cortex, orange, and cabbage juice and called by him hexuronic acid. A few weeks later Svirbely and Szent-Györgyi [101] reported the antiscorbutic activity of the latter's hexuronic acid, which was confirmed by Harris, Mills, and Innes.[102] It was now possible to bring to a swift culmination the determination of the chemical structure of the vitamin and its

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Stoff des Citronensaftes, *Z. Untersuch. Lebensm.*, 63: 1–20 (1932); and Tillmans, J., Hirsch, P., and Jackisch, J., Das Reduktionsvermögen pflanzlicher Lebensmittel und seine Beziehung zum Vitamin C. 3. Das Gehalt der verschiedenen Obst- und Gemüsearten an reduzierendem Stoff, *Z. Untersuch. Lebensm.*, 63: 241–267 (1932).

[95] Harris, L. J., and Ray, S. N., Vitamin C and the Suprarenal Cortex. II. Loss of Potency of Guinea-pig Suprarenals in Scurvy. With Notes on a Method for Determining Antiscorbutic Activity (Hexuronic Acid) by Chemical Means, *Biochem. J.*, 27: 303–310 (1933); and Birch, T. W., Harris, L. J., and Ray, S. N., A Micro-chemical Method for Determining the Hexuronic Acid (Vitamin C) Content of Foodstuffs, etc., *ibid.*, 27: 590–594 (1933).

[96] Bessey, O. A., and King, C. G., The Distribution of Vitamin C in Plant and Animal Tissues, and its Determination, *J. Biol. Chem.*, 103: 687–698 (1933); *cf.* also Musulin, R. R., and King, C. G., Metaphosphoric Acid in the Extraction and Titration of Vitamin C, *ibid.*, 116: 409–413 (1936).

[97] Emmerie, A., and van Eekelen, M., The Chemical Determination of Vitamin C with Removal of Interfering Reducing and Coloured Substances, *Biochem. J.*, 28: 1153–1154 (1934).

[98] Harris, L. J., and Ray, S. N., Diagnosis of Vitamin C Subnutrition by Urine Analysis, *Lancet* (London) I: 71–76 (1935).

[99] King, C. G., and Waugh, W. A., The Chemical Nature of Vitamin C, *Science*, 75: 357–358 (1932); and Waugh, W. A., and King, C. G., Isolation and Identification of Vitamin C, *J. Biol. Chem.*, 97: 325–331 (1932).

[100] Szent-Györgyi, A., Observations on the Function of Peroxidase Systems and the Chemistry of the Adrenal Cortex. Description of a New Carbohydrate Derivative, *Biochem. J.*, 22: 1387–1409 (1928).

[101] Svirbely, J. L., and Szent-Györgyi, A., Hexuronic Acid as the Antiscorbutic Factor, *Nature*, 129: 576, 690 (1932); and The Chemical Nature of Vitamin C, *Biochem. J.*, 26: 865–870 (1932).

[102] Harris, L. J., Mills, I., and Innes, J. R. M., The Chemical Identification of Vitamin C, *Lancet* (London) (1932) II: 235–237.

synthesis. This was greatly aided by the discovery [103] of a rich source of the substance in the ripe fruits of the red pepper and the preparation of large quantities [104] of it, by means of the easily crystallizable monoacetone derivative, discovered by v. Vargha.[105] Actually, synthesis of ascorbic acid was accomplished by Reichstein, Grüssner, and Oppenauer [106] before the correct structural formula was established. The first product was the inactive *d*-series form isolated as the acetone derivative, but later the same workers [107] synthesized the active *l*-series compound from *l*-xylosone by cyanhydrin synthesis. The correct structure of the vitamin was suggested by Euler and Martius [108] and was proved correct by the extensive experiments in Haworth's [109] laboratory in the University of Birmingham where the synthesis of the vitamin and various isomers was also accomplished. The steps in this synthesis, which proved the structure, were as follows:

$$\underset{l\text{-xylose}}{CH_2OH(CHOH)_3CHO} \rightarrow \underset{l\text{-xylosone}}{CH_2OH(CHOH)_2\cdot CO\cdot CHO} \rightarrow$$

$$\underset{\beta\text{-ketonitrile}}{CH_2OH(CHOH)_2\cdot CO\cdot CHOH\cdot CN} \xrightarrow{HOH}$$

$$\underset{\beta\text{-keto acid}}{CH_2OH\cdot(CHOH)_2\cdot CO\cdot CHOH\cdot COOH} \rightarrow$$

$$\underset{l\text{-ascorbic acid}}{CH_2OH\cdot CHOH\cdot CH\cdot COH{=}COH\cdot CO \quad (\text{with } O \text{ bridging the } CH \text{ and } COH \text{ carbons})}$$

According to King,[110] a particularly successful method of synthesis accomplished by Reichstein and Grüssner,[111] useful for the commercial synthesis of the vitamin was by means of the steps: glucose $\xrightarrow{H_2 \text{ and Pt}}$ sorbitol $\xrightarrow{B.\ xylinum}$ sorbose $\xrightarrow{\text{acetone}}$ diacetone sorbose $\xrightarrow{KMnO_4}$ diace-

[103] Svirbely, J. L., and Szent-Györgyi, A., The Chemical Nature of Vitamin C, *Biochem. J.*, 27: 279–285 (1933).

[104] Banga, I., and Szent-Györgyi, A., Large Scale Preparation of Ascorbic Acid from Hungarian Pepper (*Capsicum annuum*), *Biochem. J.*, 28: 1625–1628 (1934).

[105] v. Vargha, L., Monoacetonehexuronic Acid, *Nature*, 130: 847 (1932).

[106] Reichstein, T., Grüssner, A., and Oppenauer, R., Die Synthese der *d*-Ascorbinsäure (*d*-Form des C-Vitamins), *Helv. Chim. Acta*, 16: 561–565 (1933).

[107] Reichstein, T., Grüssner, A., and Oppenauer, R., Synthese der *d*- und *l*-Ascorbinsäure (C-Vitamin), *Helv. Chim. Acta*, 16: 1019–1033 (1933).

[108] Euler, H. von, and Martius, C., Versuche über das Gluco-reductone, *Arkiv. Kemi, Mineral. Geol.*, 11B: No. 14, 4 pages (1933).

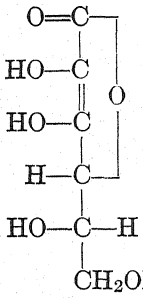
[109] Ault, R. G., Baird, D. K., Carrington, H. C., Haworth, W. N., Herbert, R., Hirst, E. L., Percival, E. G. V., Smith, F., and Stacey, M., Synthesis of *d*- and of *l*-Ascorbic Acid and of Analogous Substances, *J. Chem. Soc.*, 1419–1423 (1933).

[110] King, C. G., Vitamin C, Ascorbic Acid, *Physiol. Rev.*, 16: 238–257 (1936).

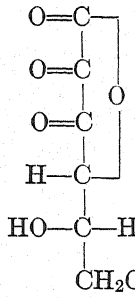
[111] Reichstein, T., and Grüssner, A., Eine Ergiebige Synthese der *l*-Ascorbinsäure (C-Vitamin), *Helv. Chim. Acta*, 17: 311–328 (1934).

tone sorbonic acid $\xrightarrow{HCl}$ *l*-sorbonic (2-keto-*l*-gulonic) acid $\xrightarrow{HCl}$ *l*-ascorbic acid (20-30 per cent yield from sorbose).

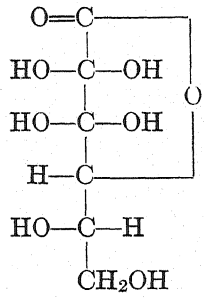
It is now established that the antiscorbutic vitamin is structurally 2-3 dienol-*l*-gulofuranolactone, which is also active in its dehydro- (2-3 diketo) form. These active lactone forms of the vitamin therefore have the following structures:



l-Ascorbic acid

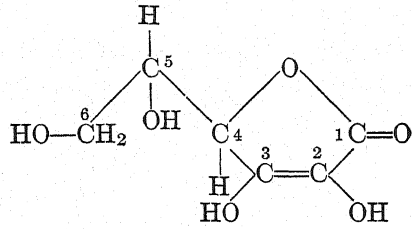


Dehydro-*l*-ascorbic acid



Hydrated form of dehydro-*l*-ascorbic acid in aqueous solution

The structure of the vitamin as a furane model, which is supported by X-ray studies, appears to be as follows:



The essential aspects of the above structures for the biological functions of the compound are not entirely clear. It might be expected that a considerable number of antiscorbutic compounds could exist or be synthesized inasmuch as there are a number of closely related pentose and hexose structures. It is known that the optically opposite *d*-series ascorbic acid is totally inactive biologically. Two pentose "ascorbic acids" possess some activity, namely, the methylated *l*-lyxose compound *l*-rhamnoascorbic acid, which has one-fifth the activity of *l*-ascorbic acid, and *d*-araboascorbic acid which has one-twentieth the activity, and *l*-glucoascorbic acid which has one-fortieth the activity. The chief acidic properties of ascorbic acid are not due to the opening of the lactone as is the case with the sugars, but are due primarily to the second enolic group on carbon atom-3. However, it is evidently not the stable ring structure characteristic

of ascorbic acid which determines its biological activity, because the lactone of 2-4-dihydroxyketobutyric acid (hydroxytetronic acid), the simplest substance of the ascorbic acid type, is not antiscorbutically active.[112]

Dehydro-*l*-ascorbic acid is a reversible oxidation product of the vitamin, the antiscorbutic activity of which was shown first by Karrer[113] and associates. The structure of the first oxidation product which has lost its activity is not established but is believed to be 2-3-dienol-*l*-gulonic acid or the corresponding diketo compound. The extensive oxidation-reduction studies on ascorbic acid carried out by Borsook[114] and associates, and by Ball,[115] leave one in doubt as to the role played by the vitamin as a redox agent in the body; according to Ball the initial oxidant begins to disappear at *p*H as low as 5.0 and has a half life of only 2 minutes at *p*H 7.25 and 38° C., yet Borsook *et al.* show that dehydroascorbic acid is rapidly reduced in the tissues. The importance of the redox properties of the vitamin in plant life seems much better established, the conditions being much more favorable there. Dehydroascorbic acid does not occur in highly acid fruits but is reported to exceed the reduced form in lettuce, string beans, and bean sprouts. The fugitive character of the vitamin under the chemical conditions existing in the animal body may explain the continued need for the vitamin in the food of species which cannot synthesize it. However, ascorbic acid is protected from oxidation in animal fluids by glutathione, proteins, and amino acids, according to Barron, Barron, and Klemperer.[116] These workers show that fresh vegetables and juices of fresh vegetables and fruits which have long been known to lose their antiscorbutic value relatively quickly lack the protective mechanisms present in animal fluids. Instead they contain "oxidases" which catalyze the reversible aerobic oxidation of the vitamin and later its destruction as shown first by Szent-Györgyi[117]

[112] Micheel, F., and Jung, F., Über die Oxytetronsäure, den einfachsten Stoffe vom Type der Ascorbinsäure, *Ber.*, 66B: 1291–1292 (1933).

[113] Karrer, P., Salomon, H., Schöpp, K., and Morf, R., Zur Kenntnis des antiskorbutischen Vitamins (Vitamin C), *Helv. Chim. Acta*, 16: 181–183 (1933).

[114] Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C., The Oxidation of Ascorbic Acid and Its Reduction in Vitro and in Vivo, *J. Biol. Chem.*, 117: 237–279 (1937).

[115] Ball, E. G., Studies on Oxidation-Reduction. XXIII. Ascorbic Acid, *J. Biol. Chem.*, 118: 219–239 (1937).

[116] Barron, E. S. G., Barron, A. G., and Klemperer, F., Studies on Biological Oxidations VII. The Oxidation of Ascorbic Acid in Biological Fluids, *J. Biol. Chem.*, 116: 563–573 (1936).

[117] Szent-Györgyi, A., On the Function of Hexuronic Acid in the Respiration of the Cabbage Leaf, *J. Biol. Chem.*, 90: 385–393 (1931).

and substantiated by Zilva,[118] and by Tauber, Kleiner, and Mishkind.[119] Tauber and associates regard the oxidase which they obtained in purified form from Hubbard squash as a specific enzyme, but Stotz, Harrer, and King[120] find that the copper content of the "enzymes" (from squash and cauliflower) is sufficient to account for the observed catalysis. The existence of a dehydroascorbic acid reductase has been suggested.[121] The practical importance of protecting the vitamin from catalytic destruction in vegetables and fruits which are to be canned has been well established. This is accomplished by a preliminary heat inactivation of the accompanying oxidase systems.

There is evidence that ascorbic acid itself exerts an effect on the catalytic activity of certain enzyme systems, activating arginase, β-amylase, and cathepsin, ferrous ascorbic acid activating papain; on the other hand, the vitamin inactivates or inhibits tyrosinase, papain, catalase, and phosphatase; cupric ascorbic acid inactivates urease, dehydroascorbic acid having like effect on α-amylase. Dehydroascorbic acid is reported[122] to dehydrate leucine with the liberation of ammonia.

The precursors of the antiscorbutic vitamin in plants and in animals which synthesize the vitamin are not known with certainty. The experiments of Ray[123] regarding mannose as an efficient precursor of the vitamin in plants and of Guha and Ghosh[124] for the same sugar in animals which synthesize their own antiscorbutic vitamin seem contrary to the structural relations between mannose and ascorbic acid. The synthesis of ascorbic acid in plants is not dependent upon photosynthesis, since it is formed in the dark and in the absence of chlorophyll, but the importance of this redox substance in subsequent photosynthetic chemical processes seems established. The site of as-

[118] Zilva, S. S., The Reversible Enzymic Oxidation of Vitamin C., *Biochem. J.*, 28: 663–666 (1934).

[119] Tauber, H., Kleiner, I. S., and Mishkind, D., Ascorbic Acid (Vitamin C) Oxidase, *J. Biol. Chem.*, 110: 211–218 (1935).

[120] Stotz, E. H., Harrer, C. J., and King, C. G., The Chemical Nature of "Ascorbic Acid Oxidase," *Science*, 86: 35 (1937); A Study of "Ascorbic Acid Oxidase" in Relation to Copper, *J. Biol. Chem.*, 119: 511–522 (1937).

[121] Kohman, E. F., and Sanborn, N. H., Dehydroascorbic Acid Reductase, *Ind. Eng. Chem.*, 29: 189–190 (1937).

[122] Euler, H. von, Karrer, P., and Zehender, F., Das Verhalten von Vitamin C (Ascorbinsäure) und anderer Reduktone gegen katheptische und andere Enzyme, *Helv. Chim. Acta*, 17: 157–162 (1934).

[123] Ray, N. S., On the Nature of the Precursor of the Vitamin C in the Vegetable Kingdom. I. Vitamin C in the Growing Pea Seedling, *Biochem. J.*, 28: 996–1003 (1934).

[124] Guha, B. C., and Ghosh, A. R., Synthesis of Ascorbic Acid (Vitamin C) by Means of Tissues *in vitro*, *Nature*, 134: 739 (1934); Biological Formation of Ascorbic Acid, *ibid.*, 135: 234 (1935).

corbic acid synthesis in animals remains to be established. The intestinal wall has been shown [125] to be a possible site, and the adrenal glands, although normally very rich in the vitamin, are eliminated as the sole site [126] because adrenalectomized dogs do not develop scurvy when supplied with adrenal cortical hormone. The tissues of animals which synthesize their own ascorbic acid are as rich in the vitamin as are those species, human and guinea pig, which require it in their food, and are enjoying a liberal intake. Normally the latter excrete the vitamin in the urine, solely in the reduced form, but the excretion continues on a scorbutic diet with the resulting depletion of the ascorbic acid in the tissues. Thus, when a dose of ascorbic acid is not followed promptly by the excretion in the urine, the result is regarded as indicating deficiency of the vitamin.

According to King,[127] the two specific roles for ascorbic acid in animal tissues which appear to be clearly established are: "(*a*) a respiratory function in serving as a hydrogen-transport agent between unidentified metabolytes and other carriers or molecular oxygen, by way of two or more oxidase enzyme systems; and (*b*) regulation of the colloidal condition of intercellular substances as shown by Wolbach and associates." [128] The pathologic changes resulting from ascorbic acid deficiency are reviewed by Wolbach.[129] The role of ascorbic acid in the organism as suggested by its cytology is reviewed by Bourne,[130] who feels that ascorbic acid is probably intimately bound up in cellular synthesis, particularly in the adrenal and anterior pituitary and perhaps the corpus luteum and interstitial cells of the gonads.

There is considerable evidence of a protective role played by ascorbic acid against certain infections and of increased requirements resulting from increased eliminations in tuberculosis, rheumatic fever, diphtheria, poliomyelitis, and pneumonia.

The International Unit of antiscorbutic vitamin is the antiscorbutic activity of 0.05 mg. of *l*-ascorbic acid, which is the International

[125] Harde, E., and Wolff, J., Sur l'origine des vitamines C chez la souris, *Compt. rend. soc. biol.*, 116: 288–290 (1934).

[126] Vars, H. M., and Pfiffner, J. J., Vitamin C and the Adrenal Gland in the Dog, *Proc. Soc. Exptl. Biol. Med.*, 31: 839–843 (1934).

[127] King, C. G., Vitamin C, Ascorbic Acid, *Physiol. Rev.*, 16: 238–262 (1936).

[128] Wolbach, S. B., and Howe, P. R., Intercellular Substances in Experimental Scorbutus, *Arch. Path. Lab. Med.*, 1: 1–24 (1926); Menkin, V., Wolbach, S. B., and Menkin, M. F., Formation of Intercellular Substance by the Administration of Ascorbic Acid (Vitamin C) in Experimental Scorbutus, *Am. J. Path.*, 10: 569–575 (1933); and Wolbach, S. B., Controlled Formation of Collagen and Reticulum: A Study of the Source of Intercellular Substance in Recovery from Experimental Scorbutus, *Am. J. Path.* (Suppl.), 9: 689–699 (1933).

[129] Wolbach, S. B., The Pathologic Changes Resulting from Vitamin Deficiency, *J. Am. Med. Assoc.*, 108: 7–13 (1937).

[130] Bourne, G., The Role of Vitamin C in the Organism as Suggested by its Cytology, *Physiol. Rev.*, 16: 442–449 (1936).

Standard. This is about one-tenth the daily dose necessary to prevent gross scorbutic lesions in a young guinea pig maintained on a scorbutogenic diet.

Certain vegetables and the citrus fruits (especially lemons, oranges, and grapefruit) are the richest sources of ascorbic acid. The following values have been reported per ounce of material, based mostly on chemical titration methods: spinach 14-27 mg., turnip greens 19 mg., red peppers 17.5 mg., green peppers 8-15 mg., oranges and lemons 11.5 mg., grapefruit 9-10 mg., raw cabbage 8-9 mg., tomatoes 4-7 mg., peas 4–7 mg., and watercress 7 mg. Extensive studies on the ascorbic acid content of fresh raw cow's milk show somewhat different ranges of values: in one study,[131] 10-15 mg. per liter, depending on the breed of cow, and 12-18 mg. per liter, depending on the stage of lactation, in another study [132] 24-30 mg. per liter independent of the ration, and in a third study [133] 20-24 mg. per liter, independent of the season. Kon and Watson [134] find that exposure of milk to daylight through glass in the presence of oxygen changes a large portion of the ascorbic acid to the reversibly oxidized form which does not reduce indophenol. The reversibly oxidized form is readily destroyed by pasteurization but not the reduced form. Human milk is reported to contain as much as five times the ascorbic acid occurring in fresh raw cow's milk and appears to fluctuate in ascorbic acid with the diet much more than does cow's milk.

The daily human requirements for *l*-ascorbic acid have been estimated as 25-30 mg. (500-600 Units) for infants and 40-50 mg. (800-1000 Units) for older children and adults. These quantities appear sufficient to keep the normal body saturated with the vitamin. Considerably less quantities protect against scurvy.

THE VITAMINS D.—Hopkins [135] apparently first recognized that some "accessory" dietary factor is concerned in the etiology of rickets. Funk [136] later classified rickets as an avitaminosis. Mellanby [137]

[131] Rasmussen, R., Guerrant, N. B., Shaw, A. O., Welch, R. C., and Bechdel, S. I., The Effects of Breed Characteristics and Stages of Lactation on the Vitamin C (Ascorbic Acid) Content of Cow's Milk, *J. Nutrition*, 11: 425–432 (1936).

[132] Riddell, W. H., Whitnah, C. H., Hughes, J. S., and Lienhardt, H. F., Influence of the Ration on the Vitamin C Content of Milk, *J. Nutrition*, 11: 47-54 (1936).

[133] Kon, S. K., and Watson, M. B., The Vitamin C Content of Cow's Milk, *Biochem. J.*, 31: 223–226 (1937).

[134] Kon, S. K., and Watson, M. B., The Effect of Light on the Vitamin C of Milk, *Biochem. J.*, 30: 2273–2290 (1936).

[135] Hopkins, F. G., The Analyst and the Medical Man, *Analyst*, 31: 385–404 (1906).

[136] Funk, C., Die Vitamine, ihre Bedeutung für Physiologie und Pathologie mit besonderer Berücksichtigung der Avitaminosen (Beri-beri, Skorbut, Pellagra, Rachitis), J. F. Bergmann, Wiesbaden (1914).

[137] Mellanby, E., An Experimental Investigation of Rickets, *Lancet*, 196: 407–412 (1919).

made the first experimental demonstration of rickets by means of a dietary deficiency of fat-soluble vitamin. It remained for McCollum [138] and his co-workers to demonstrate a distinct vitamin related to the etiology of rickets. This vitamin is known as vitamin D, although a considerable number of closely related substances exert a vitamin D effect.

Rickets is not entirely a vitamin-deficiency disease. The relative proportions of calcium and phosphorus and their concentration in the diet are important factors in the etiology of rickets. A major function of vitamin D seems to be to maintain the proper concentration of calcium and/or phosphorus in the blood, depending on the species of animal, regardless of disproportion between these elements and a deficiency of one or both of them in the diet. When the calcium and phosphorus in the diet are normal the need for vitamin D is apparently at a minimum, although there are definite species differences in this relationship. There are also species differences regarding the influence of vitamin D on the metabolism of calcium and phosphorus.

The relation of various dietary levels and ratios of calcium and phosphorus to experimental rickets and tetany in rats has been carefully and accurately established by Shohl.[139] Shohl [140] has also shown that certain experimental rachitogenic diets, having either improper ratios or improper concentrations of these elements, are rendered non-rachitogenic by the addition of either citric acid-sodium citrate or tartaric acid-sodium tartarate mixtures, whereas certain experimental non-rachitogenic diets are rendered rachitogenic by addition of ammonium chloride-ammonium carbonate mixtures of which the more important moiety is the ammonium chloride. That these are not strictly dietary acid-base balance effects is shown by the failure of mixtures of lactic, acetic, malic, malonic, and succinic acids, respectively, and their sodium salts to have the effects found for citric acid-

[138] McCollum, E. V., Simmonds, N., Becker, J. E., and Shipley, F. G., An Experimental Demonstration of the Existence of a Vitamin which Promotes Calcium Deposition, *Bull. Johns Hopkins Hosp.*, 33: 229–230 (1922).

[139] Shohl, A. T., and Wolbach, S. B.; Rickets in Rats. XV. The Effect of Low Calcium-High Phosphorus Diets at Various Levels and Ratios upon the Production of Rickets and Tetany, *J. Nutrition*, 11: 275–291 (1936); *cf.* also Brown, H. B., Shohl, A. T., Chapman, E. E., Rose, C. S., and Saurwein, E. M., Rickets in Rats. XIII. The Effect of Various Levels and Ratios of Calcium to Phosphorus in the Diet Upon the Production of Rickets, *J. Biol. Chem.*, 98: 207–214 (1932) and Bethke, R. M., Kick, C. H., and Wilder, W., The Effect of the Calcium-Phosphorus Relationship on Growth, Calcification, and Blood Composition of the Rat, *ibid.*, 98: 389–403 (1932).

[140] Shohl, A. T., The Effect of the Acid-Base Content of the Diet Upon the Production and Cure of Rickets with Special Reference to Citrates, *J. Nutrition*, 14: 69–83 (1937).

citrate and tartaric acid-tartarate mixtures, the full biochemical significance of which finding remains to be disclosed.

Reference was made to certain species differences which should be explained further. With regard to the role of vitamin D in the maintenance of normal blood calcium or phosphorus, the naturally occurring rickets in puppies, pigs, colts, and calves is usually preceded by an abnormal drop in calcium, often accompanied by tetany, rather than by a drop in inorganic phosphorus which usually precedes as well as accompanies natural rickets in chicks and infants. Both types of rickets may be produced experimentally in young rats, but the usual one is the low phosphorus type. With the exception of chicks, and to some extent infants, the dietary deficiency of mineral, either relative or absolute, is that of the element which becomes lowered in the blood. With regard to species differences in the need for vitamin D when the calcium and phosphorus in the diet are normal, we have at the one extreme the rat whose need for vitamin D is scarcely detectable when these mineral elements are normal and at the other extreme the chick which will develop severe rickets in the absence of vitamin D regardless of the calcium and phosphorus content of the diet. Other species fall somewhere in between these extremes, and therefore all have a certain absolute requirement for the vitamin. It is not without biochemical significance that the type of rickets produced in these species when vitamin D is the sole limiting factor is the type natural to the species, referred to above. Other species differences with respect to the response to different forms of vitamin D will be discussed later.

The relation of vitamin D to rickets does not mean that this vitamin is concerned solely with the metabolism of bone. Rickets is more than a bone malady. A decrease in the concentration of inorganic phosphorus or calcium in the blood is one of the first biochemical effects of vitamin D deficiency that is readily recognizable. This, together with other evidence, has led to the belief that the major biochemical function of vitamin D is in connection with the absorption and excretion of calcium and phosphorus. Other biochemical changes which have been observed are increased blood plasma phosphatase [141] and decrease in other chemical forms of phosphorus in the whole blood [142] and blood serum.[143]

[141] Kay, H. D., Plasma Phosphatase II. The Enzyme in Disease, Particularly in Bone Disease, *J. Biol. Chem.*, 89: 249–266 (1930).

[142] Stare, F. J., and Elvehjem, C. A., The Phosphorus Partition in the Blood of Rachitic and Non-Rachitic Calves, *J. Biol. Chem.*, 97: 511–524 (1932).

[143] Benjamin, H. R., and Hess, A. F., The Forms of the Calcium and Inorganic Phosphorus in Human and Animal Sera. I. Normal, Rachitic, Hypercalcemic and Other Conditions, *J. Biol. Chem.*, 100: 27–55 (1933).

The decreased concentration of nearly all forms of phosphorus in the blood which accompanies rickets affords an explanation for the general muscular weakness and instability of the nervous system which occur in this disease just as the low blood calcium which may also occur explains the tetany sometimes seen in rickets.

Many variations in the anatomical changes occurring in "rickets" have been described. These are no doubt related to the variations in blood calcium and phosphorus which are natural for the "rickets" of each species. The two extremes encountered are probably osteoporosis (accompanied by low blood calcium) and true rickets, accompanied by low blood phosphorus. Various intermediate conditions are conceivable. The problem has been carefully reviewed by Theiler.[144]

Man and animals derive relatively little of the vitamin D which they require from their food, because this vitamin does not occur abundantly in foods. This seems surprising in view of the fact that photochemical activation of provitamins D, which are apparently widely distributed and relatively abundant in plant and animal life, is the only demonstrated origin of vitamin D in nature. Indeed, land animals seem to derive most of their vitamin D by transformation of provitamin or provitamins in their own tissues, brought about through the incidence on their bodies of the proper light rays from the sun.

According to Bills[145] and co-workers, at least eight different forms of vitamin D have already been demonstrated, and a ninth is indicated. Five of these could exist naturally because they are either photochemical activation products of known substances or of substances whose existence is definitely proved. The ninth form, if proved, would make a sixth natural form, since it occurs in fish-liver oil. The various forms of vitamin D are as follows:

1. *Calciferol.* A derivative of ergosterol; producible by various forms of irradiation; can be isolated in pure crystalline form, called vitamin D_2 by the Germans; formerly regarded as the only vitamin D in nature but is now known to be absent (essentially) from cod-liver oil, although it may occur in other fish-liver oils; is the vitamin D in irradiated yeast and in the most important medicinal forms on the market (viosterol, vigantol, etc.).

[144] Theiler, A., The Osteodystrophic Diseases of Domesticated Animals. (1) The Structure of Bone; Atrophy; Osteoporosis; Osteomyelitis. (2) Rickets and Osteomalacia. (3) The Osteodystrophia Fibrosa, *Vet. J.*, 90: 143–158; 159–175; 183–206 (1934).

[145] Bills, C. E., The Multiple Nature of Vitamin D, *Cold Spring Harbor Symposia on Quantitative Biology*, 3: 328–340 (1935); New Forms and Sources of Vitamin D, *J. Am. Med. Assoc.*, 108: 13–15 (1937); and Bills, C. E., Massengale, O. N., Imboden, M., and Hall, H., The Multiple Nature of Vitamin D of Fish Oils, *J. Nutrition*, 13: 435–452 (1937).

2. *22-Dihydrocalciferol.* An irradiation derivative of synthetic 22-dihydroergosterol and called vitamin D_3 by the Germans; could exist in fish-liver oils.

3. *An irradiation derivative of 7-dehydrocholesterol.* This may be the chief vitamin in cod-liver oil and other very active fish-liver oils; the 7-dehydrocholesterol may be the chief provitamin D in milk and animal fats and in the animal skin in which vitamin D is produced by sunlight. It is this provitamin D, rather than ergosterol, as first believed, which occurs in crude cholesterol; the mistake was due to 7-dehydrocholesterol and ergosterol having the same absorption spectra.

4. *An irradiation derivative of a provitamin of unknown structure* occurring in chemically purified cholesterol. This is undoubtedly a naturally occurring vitamin, but its importance is not yet known.

5. *An irradiation product of 7-hydroxycholesterol,* an isomer produced by heating cholesterol. It is not known whether this provitamin D occurs naturally.

6. *An irradiation product of a derivative of sitosterol,* possibly 7-dehydrositosterol, since it is made from sitosterol by the same method used to produce 7-dehydrocholesterol from cholesterol. This may be the important provitamin D of higher plants, rather than ergosterol as has been believed.

7. *Cholesterilene sulfonic acid.*[146] This is a synthetic form of vitamin D made by treating cholesterol with fuller's earth.

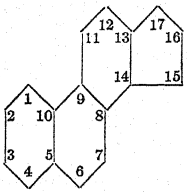
8. *A synthetic vitamin D* made by treating ergosterol with alkyl nitrites or with nitrous acid fumes, and then treating the reaction product with alkylamines, such as isopropylamine. The pure ergosterol derivative, which has great vitamin D activity, has not been isolated.

9. *A specific natural form of vitamin D* the evidence for which is a supposed different efficacy ratio for chicks and rats from that found for the other known forms of vitamin D.

The establishment of different efficacy ratios for chicks and rats of different forms of vitamin D has given the biochemist an exceptionally useful tool for ferreting out the various vitamins D. However, the final chapter in this story has not been written. By this means Bills (*loc cit.*) and co-workers have been able to differentiate the liver oil of different species of tunafish, thus introducing a new biochemical basis for taxonomy.

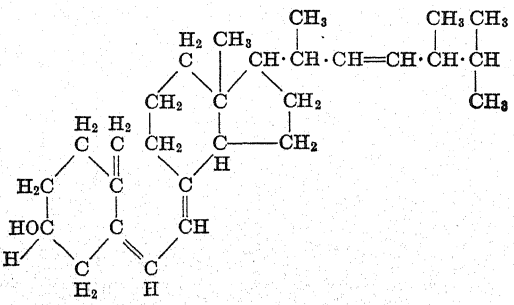
[146] Yoder, L., An Antirachitic Derivative of Cholesterol, *Science,* 80: 385–386 (1934).

Inasmuch as the vitamins D are derived only from sterols, the phenanthranecyclopentane or cholane skeleton,



Phenanthranecyclopentane (cholane) skeleton

must be the important structure in the provitamin D molecule. The important difference between ergosterol and calciferol seems to be a rupture of the C_9-C_{10} linkage of ergosterol. Calciferol is believed to have the following structure:



Calciferol (according to Windaus and Heilbron)

The vitamin D effectiveness of this product is increased [147] for chicks by hydrogenation of the double bond in the side chain. This explains in part the greater effectiveness of the cod-liver oil vitamins D which appear to be chiefly cholesterol derivatives. The cholesterol side chain is saturated.

Crystalline calciferol is a very potent drug; as little as 0.025 μg. daily, for several days, produces measurable healing of experimental rickets in a rat. The International Standard Vitamin D is an olive oil solution containing 0.0025 per cent pure crystalline calciferol. The International Unit of Vitamin D is the vitamin D activity of 1 mg. of the standard solution. Thus, the unit is the activity of 0.025 μg. of calciferol, or the activity of calciferol is 40,000 units per mg. The U. S. Pharmacopoeia, through the Vitamin Division of the U. S. Bureau of Chemistry, has provided a Reference Standard Cod Liver Oil which has been biologically assayed (on rats) against the International Standard.

[147] McDonald, F. G., The Multiple Nature of Vitamin D. III. Irradiated 22-Dihydroergosterol, *J. Biol. Chem.*, 114; *Proc. Am. Soc. Biol. Chem.*, lxv (1936).

It is obvious that the existence of vitamin D in numerous forms, of which none of the natural ones have been isolated, precludes its quantitative estimation by chemical or physical methods. Biological methods of assay are necessary, but these are subject to the usual errors of such methods. For vitamins D, in addition, there are species differences in response to the different forms, as already explained. In terms of International Units obtained by rat assay, chicks require 100 times as many units of vitamin D in the form of irradiated ergosterol as of cod-liver oil to produce normal bone mineralization. For other degrees of mineralization the ratio is less for subnormal and much greater for very hard bone.[148] This is the efficacy ratio tool referred to previously. Fortunately there seems to be little, if any, practical difference for infants between the different available forms of vitamin D. Average cod-liver oil contains 100 International Units per gram, although the U.S.P. requirement is 85 Units. Estimates regarding the daily vitamin D needs of normal infants vary from 300 to 1500 Units and for rachitic infants from 700 to 3000 Units. Toxic doses of vitamin D in the form of natural products are not probable but are producible by the very potent, synthetic calciferol-containing drugs if more than 150,000 Units are given daily. The toxicity of cod-liver oil for herbivora [149] does not reside in its vitamin content.

For a comprehensive review of some of the above and other aspects of vitamin D the student is referred to the paper by Bills.[150]

d-RIBOFLAVIN.—As the result of numerous studies culminating with those of Smith and Hendrick,[151] of Goldberger [152] and associates, as well as many other investigators, it became generally recognized that so-called vitamin B as it concerns normal growth of rats consists of some substance in addition to the antineuritic vitamin and that this substance remains after the antineuritic property of yeast and other products is destroyed by autoclaving. This factor came to

[148] Massengale, O. N., and Bills, C. E., A Quantitative Method for the Assay of Vitamin D with Chicks, *J. Nutrition*, 12: 429–446 (1936).

[149] Madsen, L. L., McCay, C. M., Maynard, L. A., Davis, G. K., and Woodward, J. C., Synthetic Diets for Herbivora, with Special Reference to the Toxicity of Cod Liver Oil, Cornell University Agr. Exp. Sta., Memoir 178: 1–53 (1935).

[150] Bills, C. E., Physiology of the Sterols, Including Vitamin D, *Physiol. Rev.*, 15: 1–97 (1935).

[151] Smith, M. I., and Hendrick, E. G., Some Nutrition Experiments with Brewer's Yeast, with Especial Reference to Its Value in Supplementing Certain Deficiencies in Experimental Rations, *U. S. Public Health Repts.*, 41: 201–207 (1926).

[152] Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., A Further Study of Butter, Fresh Beef and Yeast as Pellagra Preventives, with Consideration of the Relation of Factor P-P of Pellagra (and Black Tongue of Dogs) to Vitamin B, *U. S. Public Health Repts.*, 41: 297–318 (1926); Goldberger, J., and Lillie, R. D., A Note on an Experimental Pellagra-like Condition in the Albino Rat, *U. S. Public Health Repts.*, 41: 1025–1029 (1926).

be known as vitamin G or vitamin B_2 and, because its deficiency seemed to be accompanied by the appearance of pellagra-like skin lesions in rats, became widely accepted as the antipellagra vitamin. When the growth factor was isolated it was found to have neither antidermatitis properties for mammals nor antipellagra properties for humans, but instead marked redox properties, especially as a portion of a ferment molecule. In addition it stimulated growth of young rats and other animals and prevented various pathological conditions. However, the biochemical studies which led to the isolation of the vitamin began in the field of enzymes, not in the field of nutrition.

Banga and Szent-Györgyi [153] described a yellow water-soluble pigment accompanying an oxidation coenzyme in heart muscle. The color of the pigment, named "cytoflav," was destroyed by reducing agents and restored by atmospheric oxygen. Almost simultaneously Warburg and Christian [154] announced their discovery from bottom yeast of the yellow "oxidation" enzyme, the color of whose aqueous solution, like that of "cytoflav," was destroyed on reduction. The suggestion was made that "cytoflav" might be a decomposition product of the yellow enzyme. At the same time the occurrence of such a pigment, characterized by strong green fluorescence, in all extracts containing vitamin B_2 (G) attracted the attention of Kuhn [155] and associates who soon were able to produce convincing evidence [156] of the growth-promoting effects of the pigment when given to rats placed on so-called vitamin B_2 (G) deficient diets. Also Booher,[157] working in Dr. Sherman's laboratory in Columbia University, produced important evidence that the Bourquin-Sherman [158] units of vitamin G (B_2) representing a typical biological method for estimating the vitamin, actually measure the growth-promoting flavin content of the test substance.

[153] Banga, I., and Szent-Györgyi, A., Über Co-Fermente, Wasserstoffdonatoren und Arsenvergiftung der Zellatmung, *Biochem. Z.*, 246: 203–214 (1932).

[154] Warburg, O., and Christian, W., Über ein neues Oxydationsferment und sein Absorptionsspektrum, *Biochem. Z.*, 254: 438–458 (1932).

[155] Kuhn, R., György, P., and Wagner-Jauregg, T., Über eine neue Klasse von Naturfarbstoffen, *Ber.*, 66B: 317–320 (1933).

[156] Kuhn, R., György, P., and Wagner-Jauregg, T., Über Ovoflavin, den Farbstoff des Eiklars, *Ber.*, 66B: 576–580 (1933); Über Lactoflavin, den Farbstoff der Molke, *ibid.*, 66B: 1034–1038 (1933); Kuhn, R., and Wagner-Jauregg, T., Über die aus Eiklar und Milch isolierten Flavine, *ibid.*, 66B: 1577–1582 (1933); Kuhn, R., Rudy, H., and Wagner-Jauregg, T., Über Lacto-flavin (Vitamin B_2), *ibid.*, 66B: 1950–1956 (1933); György, P., Kuhn, R., and Wagner-Jauregg, T., Flavine und Flavoproteine als Vitamin B_2, *Z. physiol. Chem.*, 223: 241–244 (1934); Kuhn, R., and Wagner-Jauregg, T., Lacto-flavin (Vitamin B_2), aus Leber, *Ber.*, 67B: 1770–1773 (1934).

[157] Booher, L. E., The Concentration and Probable Chemical Nature of Vitamin G, *J. Biol. Chem.*, 102: 39–46 (1933); Further Studies on the Concentration and Chemical Nature of Vitamin G, *ibid.*, 107: 591–597 (1934).

[158] Bourquin, A., and Sherman, H. C., Quantitative Determination of Vitamin G (B_2), *J. Am. Chem. Soc.*, 53: 3501–3505 (1931).

In fact, the flavin isolated by Kuhn and Wagner-Jauregg from liver was stated to show an activity of 100 Bourquin-Sherman units per milligram.[159] The widespread distribution of an identical flavin in various plant and animal tissues and products was soon established and the synthesis of the natural vitamin accomplished by both Karrer [160] and Kuhn [161] and their associates. Thus the chemical structure was found to be that of a definite *iso*alloxazine having a *d*-ribose side chain, as explained in Chapter XXXI; the *iso*alloxazine ring structure of the vitamin had already been established by the synthesis of lumiflavin in 1934 by both Karrer and Kuhn.

The flavin vitamin thus has the properties of four different classes of organic compounds, those of the sugars through its pentose side chain, of the pyrimidines through its alkali-labile ring, of the azine dyes, and of the benzene derivatives, the ortho-position of the methyl groups on the benzene ring being very unusual. The sugar, attached as non-glycoside, differentiates the vitamin from the nucleosides and is regarded as accounting for its stability towards heat and acids.

Inasmuch as the natural flavin vitamin is widely distributed in nature, its earlier designation as lactoflavin, ovoflavin, hepatoflavin, etc., depending on the source from which it is isolated, has been discontinued in favor of a universal chemical designation, riboflavin (actually *d*-riboflavin). Karrer has shown that ribose may be replaced by arabinose and that either of the benzene methyls may be eliminated or replaced by ethyl without much loss in vitamin activity, but the remarkable result is that the biologically active araboflavin is the one which contains the unnatural optical form of arabinose.

Riboflavin, apparently, occurs chiefly as a monophosphoric acid ester, and carries out its functions either as the ester or as part of the yellow enzyme, of which it comprises not over 5 per cent, according to Warburg and Christian.[162] The phosphorus-free pigment functions as effectively as the ester as a dietary supplement,[163] presumably being phosphorylated in the body cells under the control of the cortical hormone (see Chapter XXXVI).

[159] Ansbacher, S., Supplee, G. C., and Bender, R. C., Lactoflavin, A Necessary Growth-Promoting Dietary Factor, *J. Nutrition*, 11: 401–409 (1936) give the potency of pure riboflavin as 150 Bourquin-Sherman units per milligram.

[160] Karrer, P., Schöpp, K., Benz, F., and Pfaehler, K., Synthesen von Flavinen, III, *Helv. Chim. Acta*, 18: 69–79; IV, *ibid.*, 18: 426–429 (1935); Euler, H. von, Karrer, P., Malmberg, M., Schöpp, K., Benz, F., Becker, B., and Frei, P., Synthese des Lactoflavins (Vitamin B_2) und andere Flavine, *ibid.*, 18: 522–535 (1935).

[161] Kuhn, R., Reinemund, K., Weygand, F., and Ströbele, R., Über die Synthese des Lactoflavins (Vitamin B_2), *Ber.*, 68B: 1765–1774 (1935).

[162] Warburg, O., and Christian, W., Über das gelbe Ferment und seine Wirkungen, *Biochem. Z.*, 266: 377–411 (1933).

[163] György, P., Growth-Promoting Activity of Lactoflavin Administered Orally and Parenterally, *Proc. Soc. Exptl. Biol. Med.*, 35: 207–209 (1936).

The differentiation of the flavin vitamin from antidermatitis food factors was accomplished by György [164] and confirmed by Harris,[165] and by Chick,[166] and Copping,[167] by Bender [168] and associates, and others. That riboflavin is not concerned with black tongue or with human pellagra was established by Birch and associates,[169] Koehn and Elvehjem,[170] and Sebrell and associates,[171] and other investigators.

Uncomplicated *d*-riboflavin deficiency produces alopecia and cataract in rats.[172] In dogs [173] a progressive disease slowly develops, characterized by loss of weight, vomiting and bloody diarrhea, flaccid muscular weakness, incoordination and decrease in the deep reflexes. According to Lepkovsky and Jukes,[174] riboflavin deficiency in chicks results in weakness, emaciation, and diarrhea, but in turkey poults there is an intense dermatitis, accompanied by slow growth and high mortality. The specific relation of these pathological conditions to the known chemical properties of riboflavin remains to be established. All higher animals are assumed to require riboflavin for the synthesis of flavoproteins which function as intermediary catalysts in vital dehydrogenation processes. Riboflavin deficiency has not been reported in man or animals under natural conditions.

As previously stated, the biological method of assay for riboflavin is the growth method, using rats, devised by Bourquin and Sherman for vitamin G (B_2). Improvements in and modification of this pro-

[164] György, P., Investigations on the Vitamin B_2 Complex. I. The Differentiation of Lactoflavin and the "Rat Antipellagra" Factor, *Biochem. J.*, 29: 741–759 (1935).

[165] Harris, L. J., Flavin and the Pellagra-Preventing Factor as Separate Constituents of a Complex Vitamin B_2, *Biochem. J.*, 29: 776–781 (1935).

[166] Chick, H., Copping, A. M., and Edgar, C. E., The Water-Soluble B-Vitamins. IV. The Components of Vitamin B_2, *Biochem. J.*, 29: 722–734 (1935).

[167] Copping, A. M., Note on the Two Types of Skin Lesions Occurring in Vitamin B_2 Deficiency in the Rat in Relation to Deficiency of Flavin and Vitamin B_6, Respectively, *Biochem. J.*, 30: 845–848 (1936).

[168] Bender, R. C., Ansbacher, S., Flanigan, G. E., and Supplee, G. C., The Influence of Dextrin and Sucrose on Growth and Dermatitis, *J. Nutrition*, 11: 391–400 (1936).

[169] Birch, T. W., György, P., and Harris, L. J., The Vitamin B_2 Complex. Differentiation of the Antiblack-Tongue and the "P.-P" Factors from Lactoflavin and Vitamin B_6 (So-Called "Rat Pellagra" Factor), I-VI, *Biochem. J.*, 29: 2830–2850 (1935).

[170] Koehn, C. J., and Elvehjem, C. A., Studies on Vitamin G (B_2) and Its Relation to Canine Black Tongue, *J. Nutrition*, 11: 67–76 (1936).

[171] Sebrell, W. H., Hunt, D. J., and Onstott, R. H., Lactoflavin in the Treatment of Canine Black Tongue, *U. S. Public Health Repts.*, 52: 235–239 (1937).

[172] Day, P. L., Darby, W. J., and Langston, W. C., The Identity of Flavin with the Cataract-Preventive Factor, *J. Nutrition*, 13: 389–399 (1937).

[173] Zimmerman, H. M., Cowgill, G. R., and Fox, J. C., Neurologic Manifestations in Vitamin G (B_2) Deficiency, *Arch. Neurol. Psychiat.*, 37: 286–306 (1937).

[174] Lepkovsky, S., and Jukes, T. H., The Response of Rats, Chicks and Turkey Poults to Crystalline Vitamin G (Flavin), *J. Nutrition*, 12: 515–526 (1936).

cedure have been suggested by Day,[175] Page,[176] Hamilton and Mitchell,[177] and by Cook, Clarke and Light.[178] Jukes[179] has described a method using chicks. The marked fluorescence of riboflavin solutions has provided a physical method of assay for this substance. As developed by Supplee[180] and associates, the fluorescent emissions under black light (ultraviolet) of unknowns compared with a standard riboflavin solution make possible the detection of concentrations as low as 0.5 μg. per ml. Whitnah *et al.*[181] give a similar procedure for estimating riboflavin in milk.

The concentration of riboflavin in foods, based on biological assays as summarized by Eddy and Dalldorf (*loc. cit.*), ranges below 50 Bourquin-Sherman units per ounce for most foods except cheese and turnip and beet greens which range between 50 and 100 units per ounce, and for dried milk which may contain over 100 units per ounce, and for meats and dried yeast which contain over 200 units per ounce. These general figures may be translated into actual concentrations of riboflavin, if it be assumed that 0.6 to 1.0 μg. of the vitamin has 1 unit activity.

VITAMIN E (α-TOCOPHEROL).—Vitamin E was discovered by Evans and Bishop[182] in a study of the estrous cycle of female rats fed for relatively long periods upon synthetic diets with an ample provision for all the previously known vitamins. They found that reproductive failure occurs which is very characteristic in its nature. Although ovulation takes place, and the embryos are implanted and begin to develop normally, the placental function fails before parturition, with the accompanying death and resorption of the implanted embryos. Reproduction in the sense of failure for young to appear may occur as a result of many other dietary deficiencies. It is to be

[175] Day, P. L., Vitamin G in Beef and Veal, *J. Home Econ.*, 23: 657–661 (1931).

[176] Page, J. W., An Improvement in Experimental Method for Investigation of Vitamin G, *Proc. Soc. Exptl. Biol. Med.*, 30: 87–88 (1932).

[177] Hamilton, T. S., and Mitchell, H. H., A Modification of the Sherman Method of Studying the Multiple Nature of Vitamins, with an Application to Vitamin G, *J. Nutrition*, 10: 117–128 (1935).

[178] Cook, C. A., Clarke, M. F., and Light, A. E., Biological Assays for Flavin and Dermatitis Factor(s), *Science*, 85: 503–504 (1937).

[179] Jukes, T. H., Biological Assay of Lactoflavin with Chicks, *J. Nutrition*, 14: 223–233 (1937).

[180] Supplee, G. C., Ansbacher, S., Flanigan, G. E., and Hanford, Z. M., The Fluorometric Estimation of Lactoflavin, *J. Dairy Sci.*, 19: 215–220 (1936).

[181] Whitnah, C. H., Kunerth, B. L., and Kramer, M. M., A Rapid Method for the Determination of Lactoflavin in Milk, *J. Am. Chem. Soc.*, 59: 1153–1154 (1937).

[182] Evans, H. M., and Bishop, K. S., On the Existence of a Hitherto Unrecognized Dietary Factor Essential for Reproduction, *Science*, 56: 650–651 (1922); On the Relations between Fertility and Nutrition, I-IV, *J. Metabolic Research*, 1: 319–333; 335–356 (1922); 3: 201–231; 233–316 (1923).

emphasized, therefore, that vitamin E deficiency is at present detected in female mammals chiefly through the special histological technic developed by Evans and Bishop.

The physiological effects of vitamin E deficiency are manifested in other ways. A type of male sterility [183] also develops, although much more slowly than the reproductive failures in the female. The effects on the male may be permanent, but this is not likely to be the case for female animals. Evans and Burr [184] studied a form of paralysis in suckling young rats nursed by mothers on certain diets. This condition can be cured by changing the diet to natural food, or prevented by foods rich in vitamin E or by vitamin-E-rich concentrate prepared from wheat germ oil. It would appear, therefore, that vitamin E performs some necessary function in the mammalian growth *postpartum* as well as in embryo. This is also true for birds, as shown by the work of Adamstone [185] on chicks, both in embryo and after hatching. The developing chick embryo deprived of vitamin E exhibits marked proliferation of the cells of certain tissues, resulting in early death. Chicks develop loss of muscular control after one month on the vitamin-E-free diet, with accompanying changes in the cerebellum. There are also degenerative lesions in the viscera characterized by profuse cell proliferation.

A growth-stimulating effect of vitamin E concentrates on rats, after having grown at a normal rate for 6 to 8 months, has been reported by Evans [186] and by Emerson and Evans.[187] Olcott and Mat-

[183] Mattill, H. A., Carman, J. S., and Clayton, M. M., The Nutritive Properties of Milk. III. The Effectiveness of the X Substance in Preventing Sterility in Rats on Milk Rations High in Fat, *J. Biol. Chem.*, 61: 729–740 (1924); Mason, K. E., A Histological Study of Sterility in the Albino Rat Due to a Dietary Deficiency, *Proc. Natl. Acad. Sci., U. S.*, 11: 377–382 (1925); Evans, H. M., Invariable Occurrence of Male Sterility with Dietaries Lacking Fat Soluble Vitamine E, *ibid.*, 11: 373–377 (1925); Mason, K. E., Testicular Degeneration in Albino Rats Fed a Purified Food Ration, *J. Exptl. Zool.*, 45: 159–229 (1926); Mattill, H. A., The Relation of Vitamins B and E to Fertility in the Male Rat, *Am. J. Physiol.*, 79: 305–315 (1927); Evans, H. M., and Burr, G. O., The Antisterility Vitamine Fat Soluble E, Memoirs of the University of California, Vol. 8 (1927); and Adamstone, F. B., and Card, L. E., The Effects of Vitamin E Deficiency on the Testis of the Male Fowl (*Gallus domesticus*), *J. Morphol.*, 56: 339–359 (1934).

[184] Evans, H. M., and Burr, G. O., Development of Paralysis in the Suckling Young of Mothers Deprived of Vitamin E, *J. Biol. Chem.*, 76: 273–297 (1928).

[185] Adamstone, F. B., The Effects of Vitamin E Deficiency on the Development of the Chick, *J. Morphol.*, 52: 47–90 (1931); The Effects of Severe and Prolonged Vitamin E Deficiency in the Chick, *Anat. Record*, 60: Suppl. 36–37 (1934); A Possible Relation of Vitamin E to Unrestricted Cell Division, *Science*, 80: 450 (1934).

[186] Evans, H. M., Relation of Vitamin E to Growth and Vigor, *J. Nutrition*, 1: 23–28 (1928).

[187] Emerson, Gladys A., and Evans, H. M., The Effect of Vitamin E Deficiency Upon Growth, *J. Nutrition*, 14: 169–178 (1937).

till [188] find that this is definitely an effect occurring in late adolescent life in this species, growth stimulation occurring at an earlier age in males than in females. Martin [189] reports some success in separating the growth effect and the antisterility effect of vitamin E concentrates by fractional solubility methods.

The established effect of numerous hormones on various aspects of the reproductive phenomena has stimulated considerable study of the effect of vitamin E deficiency on the endocrine glands. The studies involving the pituitary and the pituitary sex hormones have been contradictory, investigators, such as Olcott and Mattill,[190] working with highly concentrated preparations, finding no relationship. However, the thyroid gland of female rats having proved sterility as the result of vitamin E deprivation is markedly hypoplastic, according to Miss Singer;[191] this is not affected by iodine administration.

Attempts to isolate vitamin E began with Evans and Burr,[192] who prepared a sterol-free fraction from the unsaponifiable ether extract of wheat germ oil, which, after steam distillation at 180° C., 50 mm. pressure, and several distillations *in vacuo*, yielded the most active material at 225-230° C. (0.01 mm.), and represented about 0.25 per cent of the original oil. It cured vitamin E deficiency sterility in female rats in single doses of 5 mg. It was reported to be inactivated on acetylation, a result which must have been in error, judging from the later work of Olcott (*vide infra*).

The almost invariable association of vitamin E with antioxidant material in natural foodstuffs was pointed out by Cummings and Mattill.[193] When Olcott and Mattill [194] applied the Evans-Burr fractionation scheme to the vitamin E extracts of lettuce, they succeeded in separating the antioxidant and the vitamin by phase separation between 92 per cent MeOH and petroleum ether, the vitamin going into the epiphasic layer. The antioxidant, isolated in crystalline form, proved to be a hydroxyphenolic compound, not identified specifically.

[188] Olcott, H. S., and Mattill, H. A., Vitamin E and Growth, *J. Nutrition*, 14: 305–315 (1937).

[189] Martin, G. J., Vitamin E., *J. Nutrition*, 13: 679–685 (1937).

[190] Olcott, H. S., and Mattill, H. A., Vitamin E. I. Some Chemical and Physiological Properties, *J. Biol. Chem.*, 104: 423–435 (1934).

[191] Singer, Eleanor, Effects of Vitamin E Deficiency in the Thyroid Gland of the Rat, *J. Physiol.*, 87: 287–290 (1936).

[192] Evans, H. M., and Burr, G. O., The Antisterility Vitamine Fat Soluble E, XVIII. Steps in the Isolation and Concentration of Vitamine E, Memoirs of the University of California, Vol. 8, pp. 129–146, University of California Press, Berkeley (1927).

[193] Cummings, M. J., and Mattill, H. A., The Autooxidation of Fats with Reference to their Destructive Effect on Vitamin E, *J. Nutrition*, 3: 421–432 (1931).

[194] Olcott, H. S., and Mattill, H. A., The Unsaponifiable Lipids of Lettuce. II. Fractionation. III. Antioxidant, *J. Biol. Chem.*, 93: 59–64, 65–70 (1931).

This result corresponds very well with the finding of Fernholz [195] that the α-tocopherol of Evans, Emerson, and Emerson [196] decomposes on distillation to yield durohydroquinone. When Olcott [197] examined the antioxidant properties of the tocopherols, of which the α-form is regarded by the California workers as either vitamin E or very closely resembling it, all the forms as well as their allophanates proved to be highly antioxogenic, although not equally so. The earlier separation of antioxidant from the vitamin is explained as possibly due to the isolation of the vitamin as the ester, instead of the free hydroxyl compound. However, the vitamin E concentrates obtained at that time may be judged as less than 2 per cent as active biologically as is α-tocopherol or as were the later concentrates prepared by Olcott and Mattill [198] from wheat-germ and cottonseed oils.

These later concentrates boiled between 195° and 220° C. (0.1 mm.), and their activity at 5-mg. doses was not destroyed by hydrogenation under pressure, by acetylation, or benzoylation, but was destroyed by ozone, perbenzoic acid, potassium amide, and potassium ethylate. Chlorinated and brominated concentrates, which were inactive, could be reactivated by dehalogenation. The activity of certain esters suggests the occurrence of the vitamin in nature in both free and ester forms.

Olcott [199] was the first to notice a characteristic absorption maximum at 294 mμ of vitamin E concentrates. This was confirmed by Martin *et al.*,[200] and by Drummond [201] and associates. Later several types of evidence were advanced by Olcott [202] against the view that

[195] Fernholz, E., The Thermal Decomposition of α-Tocopherol, *J. Am. Chem. Soc.*, 59: 1154–1155 (1937).

[196] Evans, H. M., Emerson, O. H., and Emerson, G. A., The Isolation from Wheat Germ Oil of an Alcohol, α-Tocopherol, Having the Properties of Vitamin E, *J. Biol. Chem.*, 113: 319–332 (1936); Emerson, O. H., Emerson, G. A., and Evans, H. M., The Isolation from Cottonseed Oil of an Alcohol Resembling α-Tocopherol from Wheat-Germ Oil, *Science*, 83: 421 (1936).

[197] Olcott, H. S., and Emerson, O. H., Antioxidants and the Autooxidation of Fats. IX. The Antioxidant Properties of the Tocopherols, *J. Am. Chem. Soc.*, 59: 1008–1009 (1937).

[198] Olcott, H. S., and Mattill, H. A., Vitamin E. I. Some Chemical and Physiological Properties, *J. Biol. Chem.*, 104: 423–435 (1934).

[199] Olcott, H. S., Some Chemical Properties of Vitamin E, *J. Biol. Chem.*, 105: *Proc.*, lxv (1934); Vitamin E. II. Stability of Concentrates Toward Oxidizing and Reducing Agents, *ibid.*, 107: 471–474 (1934).

[200] Martin, A. J. P., Moore, T., Schmidt, M., and Bowden, F. P., Absorption Spectrum of Vitamin E, *Nature*, 134: 214 (1934).

[201] Drummond, J. C., Singer, Eleanor, and MacWalter, R. J., A Study of the Unsaponifiable Fraction of Wheat-Germ Oil with Special Reference to Vitamin E, *Biochem. J.*, 29: 456–471 (1935); Further Observations on the Constituents of the Unsaponifiable Fraction of Wheat-Germ Oil with Particular Reference to Vitamin E, *ibid.*, 29: 2510–2521 (1935).

[202] Olcott, H. S., Vitamin E. III. Evidence for the Presence of a Hydroxyl Group. The Biological Utilization of Esters. Absorption Spectrum, *J. Biol. Chem.*, 110: 695–701 (1935).

this absorption band is a property of the vitamin, and the suggestion was made that this is the property of the accompanying antioxidant. However, α-tocopherol is characterized by a definite band with a maximum at 298 mμ. Since this also resembles the absorption spectra of hydroquinone, as pointed out by Olcott and Emerson (*loc. cit.*), and since Fernholz (*loc. cit.*) has found durohydroquinone to be a definite part of α-tocopherol, probably in ether linkage with an unsaturated aliphatic chain, the explanation of these apparently divergent results regarding absorption, antioxidant, and vitamin properties would seem to be that the vitamin activity of the apparently complex molecule is not primarily concerned with its antioxidant component, which can be separated from the complex, although its antioxidant properties and spectroscopic absorption band are definitely those of the quinone part of the structure. An aliphatic, approximately C_{20} monounsaturated alcohol which possesses vitamin E activity has been derived [203] from tocopherols. Indication that the quinone part of the vitamin has biological significance is seen in the finding of John [204] that one of the other tocopherols has a cumohydroquinone ring, rather than the duro structure of α-tocopherol. This isomer has one-fourth to one-half the vitamin E activity of α-tocopherol. It is apparently identical with the β-tocopherol of Emerson *et al.*[205]

It seems anomalous that vitamin E should be regarded as especially sensitive to oxidation when it carries as a part of its structure one of the most active antioxidants known. It must be acknowledged, however, that spectroscopic absorption intensities and antioxidant properties can be determined with much greater quantitative precision than vitamin E activity. Vitamin E assays give only rough approximations, even when carried out by the best procedure now available.[206]

Vitamin E activity has been demonstrated in animal tissues and products such as muscle meat, adipose tissue, viscera, and in milk and eggs, probably originating from the food.[207] Synthesis of the vitamin is largely, if not exclusively, a function of plants, certain oil-bearing seeds or parts of seeds being relatively rich in this vitamin, notably

[203] Todd, A. R., Bergel, F., Waldman, H., and Work, T. S., Constituents of Vitamin E Concentrates from Rice and Wheat-Germ Oils, *Nature*, 140: 361–362 (1937).

[204] John, W., Über das Cumo-tokopherol, einen neuen Faktor der Vitamin E-Gruppe, *Z. physiol. Chem.*, 250: 11–24 (1937).

[205] Emerson, O. H., Emerson, G. A., Mohammad, A., and Evans, H. M., The Chemistry of Vitamin E. Tocopherols from Various Sources, *J. Biol. Chem.*, 122: 99–107 (1937).

[206] Palmer, L. S., Biological Assay of Vitamin E, *Ind. Eng. Chem., Anal. Ed.*, 9: 427–429 (1937).

[207] If all species of higher animals require vitamin E, the opportunity to test its synthesis by animals is afforded in experiments of Wilson, Thomas, and Cannon [*J. Dairy Science*, 18: 431–432 (1935)], who have reared several generations of goats on rations shown by rat tests to be free from the vitamin.

wheat-germ, cottonseed, palm seed, peanuts, alfalfa seed, and lettuce seed. The oils from rice, barley, oats, and soybeans appear to contain the vitamin but not in such high concentrations as that from wheat. Results with corn oil and olive oil have been contradictory. Green leaves, such as alfalfa or lettuce (fresh or dry), are good sources of vitamin E. Hathaway and Davis [208] found 20 to 25 per cent alfalfa meal, or wheat bran, wheat shorts, linseed-oil meal, cottonseed meal, white hominy feed, white or yellow corn, or red kafir corn as sole source of vitamin E during the gestation period of female rats of proved vitamin E sterility, to be adequate for normal reproduction. The same workers found that artificial drying of alfalfa tended to preserve its vitamin E to a greater degree than did field curing.

VITAMIN B_6.—When György [209] demonstrated that flavin failed to prevent the dermatitis in rats which had been regarded as one of the specific effects of vitamin G (B_2) deficiency, he proposed that the "rat-antipellagra" factor be called vitamin B_6. The existence of such a vitamin is generally accepted as the result of the further studies by György,[210] Chick, Copping, and Edgar,[211] and Copping.[212] The Lister Institute workers had previously designated an apparently identical substance "factor Y." The B_6 deficiency dermatitis is described by them as of the florid type. It is this type appearing bisymmetrically on the rats which resembles human pellagra, although of different etiological origin. It is called rat acrodynia to differentiate it from pellagra.

Knowledge of the chemistry of vitamin B_6 is limited largely to general properties such as the following. It is extracted from natural sources by 80 per cent alcohol, it diffuses through cellophane membranes, is stable to both heat (autoclave) and alkali (*p*H 9.0), is not

[208] Hathaway, I. L., and Davis, H. P., The Vitamin E Content of Certain Dairy Feeds, Nebraska Agr. Exp. Sta. Research Bull. 73 (1934); Hathaway, I. L., Davis, H. P., and Graves, R. R., The Vitamin A and the Vitamin E Content of Field-Cured and Artificially Cured Alfalfa Hay, Nebraska Agr. Exp. Sta. Research Bull. 62 (1932).

[209] György, P., Vitamin B_2 and the Pellagra-like Dermatitis in Rats, *Nature*, 133: 498–499 (1934).

[210] György, P., Investigations on the Vitamin B_2 Complex. I. The Differentiation of Lactoflavin and the "Rat Antipellagra" Factor, *Biochem. J.*, 29: 741–759 (1935); II. The Distribution of Lactoflavin and of the "Pellagra-Preventing Factor" (Vitamin B_6) in Natural Products of Animal Origin, *ibid.*, 29: 760–766 (1935); III. The Inactivation of Lactoflavin and Vitamin B_6 by Visible Light, *ibid.*, 29: 767–775 (1935).

[211] Chick, H., Copping, A. M., and Edgar, C. E., The Water-Soluble B-Vitamins. IV. The Components of Vitamin B_2, *Biochem. J.*, 29: 722–734 (1935).

[212] Copping, A. M., The Water-Soluble B-Vitamins. V. Note on the Two Types of Skin Lesion Occurring in Vitamin B_2 Deficiency in Relation to Deficiency of Flavin and Vitamin B_6, Respectively, *Biochem. J.*, 30: 845–848 (1936); VI. Flavin and Vitamin B_6 in Cereals, *ibid.*, 30: 849–856 (1936).

adsorbed by fuller's earth at *p*H 1.0, is not precipitated by lead acetate at either *p*H 4.0 or 8.0, and is light sensitive, especially to ultraviolet rays. The latter fact was established by Hogan and Richardson,[213]

The probability that vitamin B_6 must be regarded as necessary for growth of rats is emphasized by Gorter.[214]

According to Sjollema [214a] vitamin B_6 is isoleucine. Lepkovsky [214b] has reported the isolation of the crystalline vitamin.

Vitamin B_6 has been demonstrated in yeast, some strains apparently being richer than others, and in whole wheat and corn where it is concentrated chiefly in the germ and bran; also in heart muscle, veal, beef, and fish muscle, chicken muscle and gizzard, codfish liver, and cow's milk. György [215] found human milk and cow's milk to be equally rich in vitamin B_6.

PELLAGRA-PREVENTIVE (P.P.) FACTOR.—The establishment of a distinct vitamin-deficiency etiology for rat acrodynia raised the question of the relation between pellagra dermatitis and pellagra-like symptoms in the rat, dog, and chick. For some time so-called "rat pellagra" and black tongue in dogs had been accepted as analogs of human pellagra, and more recently [216] similar manifestations in chicks had been produced.

The first published evidence of a distinct pellagra-preventive vitamin, probably identical with the anti-black tongue and anti-chick "pellagra" factors, but distinct from vitamin B_6, was that of Birch, György, and Harris.[217] The differentiation of B_6 from the human

[213] Hogan, A. G., and Richardson, L. R., The Effect of Ultra-Violet Rays on the Dermatitis Preventing Vitamin, Missouri Agr. Exp. Sta. Res. Bull. 178, pp. 1–18 (1932); Differentiation of the Antidermatitis Factor, *Science*, 83: 17–18 (1936); Richardson, L. R., and Hogan, A. G., Skin Lesions of the Rat Associated with the Vitamin B Complex, Missouri Agr. Exp. Sta. Research Bull. 241, pp. 1–36 (1936).

[214] Gorter, F. J., Differentiation of the Growth Promoting Factors in Yeast Which are Related to Rat-Pellagra, *Arch. néerland. physiol.*, 21: 538–553 (1936).

[214a] Sjollema, B., Isoleucine als anti-dermatitis Principe, *Tijdschr. Diergeneesk.*, 64: 986–988 (1937).

[214b] Lepkovsky, S., Crystalline Factor I, *Science*, 87: 169–170 (1938).

[215] György, P., Quantitative Estimation of Lactoflavin and of Vitamin B_6 in Cow's Milk and in Human Milk, *Proc. Soc. Exptl. Biol. Med.*, 35: 204–207 (1936).

[216] Ringrose, A. T., Norris, L. C., and Heuser, G. F., The Occurrence of a Pellagra-like Syndrome in Chicks, *Poultry Sci.*, 10: 166–177 (1931); Kline, O. L., Keenan, J. A., Elvehjem, C. A., and Hart, E. B., The Use of the Chick in Vitamin B_1 and B_2 Studies, *J. Biol. Chem.*, 99: 295–307 (1932); Elvehjem, C. A., and Koehn, C. J., Jr., Studies on Vitamin B_2 (G). The Non-Identity of Vitamin B_2 and Flavins, *ibid.*, 108: 709–728 (1935); Ansbacher, S., Supplee, G. C., and Bender, R. C., Pellagra-like Syndrome in Chicks, *J. Nutrition*, 11: 529–535 (1936).

[217] Birch, T. W., György, P., and Harris, L. J., The Vitamin B_2 Complex. Differentiation of the Antiblack-Tongue and the "P.-P" Factors from Lactoflavin and Vitamin B_6 (So-Called "Rat Pellagra" Factor), I-VI, *Biochem. J.*, 29: 2830–2850 (1935).

"P.P." factor was deductive; maize and molasses, known to be outstanding pellagra-producing dietary constituents, were shown to be rich in vitamin B_6. An experimental black-tongue-producing diet caused no abnormalities in rats, whereas dogs fed a typical maize-containing black-tongue-producing diet, rich in B_6, developed the usual symptoms. Similar results, using pellagra-producing foods, were obtained by Dann,[218] who also reported that clinical cases of pellagra were not benefited by riboflavin. More conclusive evidence was obtained by Koehn and Elvehjem[219] that the chick defect is distinct from that produced by B_6 deficiency and more analogous to black tongue in dogs; these workers prefer to retain the older terminology, G (B_2), for this vitamin, although more general usage applies it to riboflavin. Lepkovsky and Jukes[220] have referred to it as the "filtrate factor" and also as "factor 2." Jukes presented arguments, based on relative distribution studies, for a distinction between "chick-pellagra"-preventive vitamin and the human "P.P." factor. However, Fouts, Lepkovsky, Helmer, and Jukes[221] reported successful treatment of human pellagra with the "filtrate factor." Thus several independent experimental studies point strongly to an identical vitamin-deficiency etiology being involved in chick "pellagra," black tongue, and human pellagra.

Attempts to concentrate the antipellagra vitamin have accompanied many of the studies referred to above. The most successful of these has been by Elvehjem[222] and associates, who appear to have brought

[218] Dann, W. J., The Vitamin G Complex. I. The Non-Identity of Rat Dermatitis Due to Vitamin B_6 Deficiency and the Dermatitis of Human Pellagra, *J. Nutrition*, 11: 451–462 (1936).

[219] Koehn, C. J., Jr., and Elvehjem, C. A., Studies on Vitamin G (B_2) and Its Relation to Canine Black Tongue, *J. Nutrition*, 11: 67–76 (1936); Further Studies on the Concentration of the Antipellagra Factor, *J. Biol. Chem.*, 118: 693–699 (1937).

[220] Lepkovsky, S., and Jukes, T. H., The Effect of Some Reagents on the "Filtrate Factor" (A Water-Soluble Vitamin Belonging to the Vitamin B Complex and Preventing a Dietary Dermatitis in Chicks), *J. Biol. Chem.*, 114: 109–116 (1936); Jukes, T. H., and Lepkovsky, S., The Distribution of the "Filtrate Factor" (A Water-Soluble Vitamin Belonging to the Vitamin B Complex and Preventing a Dietary Dermatitis in Chicks) in Certain Feedingstuffs, *ibid.*, 114: 117–121 (1936); Jukes, T. H., Further Observations on the Assay, Distribution, and Properties of the Filtrate Factor, *ibid.*, 117: 11–20 (1937); Lepkovsky, S., and Jukes, T. H., Fractionation of the Vitamin G Complex, *ibid.*, 119: *Proc.* lx (1937).

[221] Fouts, P. J., Lepkovsky, S., Helmer, O. M., and Jukes, T. H., Successful Treatment of Human Pellagra with the "Filtrate Factor," *Proc. Soc. Exptl. Biol. Med.*, 35: 245–247 (1936).

[222] Elvehjem, C. A., and Koehn, C. J., Studies on Vitamin B_2 (G). The Non-Identity of Vitamin B_2 and Flavins, *J. Biol. Chem.*, 108: 709–728 (1935); Koehn, C. J., and Elvehjem, C. A., Further Studies on the Concentration of the Antipellagra Factor, *ibid.*, 118: 693–699 (1937); Elvehjem, C. A., Madden, R. J., Strong, F. M., and Woolley, D. W., Relation of Nicotinic Acid and Nicotinic Acid Amide to Canine Black Tongue, *J. Am. Chem. Soc.*, 59: 1767–1768 (1937).

the search to a successful culmination in the isolation of nicotinic acid amide from antipellagra (chick) and anti-black-tongue concentrates from liver extract, this well-known pyridine compound and also the free acid showing marked black-tongue-curing as well as prophylactic properties. Fouts and associates [223] have reported improvement in four pellagra patients following the daily administration of 0.5–1.0 gram nicotinic acid, indicating that this long-known substance is one of the components of their "filtrate factor." Dann [224] states that nicotinic acid prevents black tongue but not chick dermatitis. On the whole these findings relate the fundamental biochemical properties of dietary nicotinic acid to enzyme actions in the body because of the recent establishment of nicotinic acid amide as the hydrogen carrier in a cozymase nucleotide composed of nicotinic acid amide, ribose, metaphosphoric acid, and adenine. The pyridine component is believed to enter the compound through a molecule of ribose.[225]

The discovery of the identity of the "P.P." factor with a known organic compound [226] should permit the clinician to determine with much greater assurance the real complicating deficiencies and associated infections long regarded as a part of naturally occurring pellagra. Chemical methods of assay may be expected which can be applied to the many food products which have been tested for pellagra-preventing properties by the workers of the United States Public Health Service, beginning with the pioneer work of Goldberger, who postulated a vitamin-deficiency etiology of the disease. The summary of their findings is given by Eddy and Dalldorf.[227]

VITAMIN B_4.—Reader [228] postulated the existence of another vitamin resembling B_1 and required for growth of young rats. She first called the vitamin B_3 but later [229] changed the designation to B_4. Besides lack of growth, the symptoms described were general weakness, swollen paws, spastic gait, and lack of coordination, which developed after the young rats were cured of polyneuritis produced by a synthetic diet containing autoclaved yeast autolyzate as source of the necessary water-soluble vitamins. Belief in the existence of this vita-

[223] Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H., Treatment of Human Pellagra with Nicotinic Acid, *Proc. Soc. Exptl. Biol. Med.*, 37: 405–407 (1937).

[224] Dann, W. J., Nicotinic Acid and Vitamin B_2, *Science*, 86: 616–617 (1937).

[225] Schlenk, F., and Euler, H., von, Cozymase, *Naturwissenschaften*, 24: 794–795 (1936).

[226] One recalls with interest that the crystalline "Beri-beri Vitamine" of Funk turned out to be largely nicotinic acid. Funk may have to be credited with the isolation of the first vitamin, although its correct function was not at that time established.

[227] Eddy, W. H., and Dalldorf, G., The Avitaminoses, pp. 158–159, The Williams and Wilkins Company, Baltimore (1937).

[228] Reader, V., A Second Thermolabile Water-Soluble Accessory Factor Necessary for the Nutrition of the Rat, *Biochem. J.*, 23: 689–694 (1929).

[229] Reader, V., The Assay of Vitamin B_4, *Biochem. J.*, 24: 1827–1831 (1930).

min was furthered by isolation [230] of biologically active crystalline material from yeast extracts. Ten micrograms daily of this material produced cures in adult rats. Its apparent distinction from the antineuritic vitamin was in part its adsorption by charcoal at highly acid *p*H in contrast to the antiberiberi vitamin which is adsorbed at neutrality. A method for large-scale production of B_4 concentrates was later described by the Oxford University workers.[231]

Support for the existence of this vitamin came also from Moore [232] and associates and from the University of Wisconsin group,[233] which is, at present, the most vigorous proponent of this factor. Chicks fed from hatching on a ration consisting of casein, dextrin, salt mixture, cod-liver oil, and dried yeast (the last comprising 8 per cent of the ration) developed in a few weeks a spastic paralysis which could be cured with vitamin B_4 preparation made from pork liver by the procedure of the Oxford laboratories. Later they [234] reported somewhat greater heat stability of the antineuritic vitamin at 100° C. than of vitamin B_4 in moist liver or in natural grain ration; this provided the procedure adopted for rendering foods free from B_4 in studies of the distribution of the vitamin in natural food products. This procedure was improved [235] by using purified casein, reducing the yeast to 2 per cent, and including 2 per cent each of autoclaved liver residue and a liver extract and 5 per cent of a water-extracted lung tissue, each to provide other essential factors for chicks. With this diet adequate B_4 was provided by 15 per cent of peanuts, brain tissue, or kidney. This list was later [236] extended to include the following foods and their minimum protective levels: dried grass, less than 10 per cent; wheat germ, 15 per cent; Crisco, less than 20 per cent; pork liver, 24 per cent; white corn and hulled oats, 32 per cent; wheat and yellow corn, 40 per

[230] Barnes, H., O'Brien, J. R., and Reader, V., Crystalline Preparation with Vitamin B_4 Activity, *J. Physiol.*, 76: 8(P) (1932); Vitamin B_4, *Biochem. J.*, 26: 2035–2040 (1932).

[231] Kinnersley, H. W., O'Brien, J. R., Peters, R. A., and Reader, V., Large Scale Preparations of Vitamin B_1 and Vitamin B_4 Concentrates, *Biochem. J.*, 27: 225–231 (1933).

[232] Moore, C. U., Plymate, H. B., and Andrew, B. J., Studies on the B Vitamins. III. Evidence of a Third Vitamin B Factor in Yeast (B_4) etc., *Am. J. Physiol.*, 102: 581–592 (1932).

[233] Keenan, J. A., Kline, O. L., Elvehjem, C. A., Hart, E. B., and Halpin, J. G., New Nutritional Factors Required by the Chick, *J. Biol. Chem.*, 103: 671–685 (1933).

[234] Keenan, J. A., Kline, O. L., Elvehjem, C. A., and Hart, E. B., The Stability of Vitamins B (B_1), G (B_2), and B_4, *J. Nutrition*, 9: 63–74 (1935).

[235] Kline, O. L., Bird, H. R., Elvehjem, C. A., and Hart, E. B., An Improved Synthetic Ration for Vitamin B_4 Studies, *J. Nutrition*, 11: 515–528 (1936).

[236] Kline, O. L., Bird, H. R., Elvehjem, C. A., and Hart, E. B., The Distribution of Vitamin B_4 in Some Plant and Animal Products, *J. Nutrition*, 12: 455–460 (1936).

cent. Some of the minimum protective levels seem very surprising. One wonders why such precautions are necessary to purify the basal ration and include small percentages of processed materials when such high concentrations of natural products are minimum levels for protection against the B_4 deficiency paralysis.

Doubt was raised regarding the necessity of B_4 for rats, and even of its existence, when Waterman and Ammerman [237] demonstrated continuous normal growth of rats receiving an ample daily supplement of crystalline antineuritic vitamin to a ration on which B_4 deficiency should have developed. Like results,[238] obtained by the Oxford workers using their crystalline antineuritic vitamin, raised somewhat similar doubts in their own minds. Because of these results reasonable doubt is permissible regarding the correct interpretation to be placed on the evidence so far published for this special antiparalysis vitamin. However, Elvehjem and Arnold [239] and Kline, Elvehjem, and Hart [240] report that young growing rats will develop typical B_4 deficiency symptoms when fed their improved B_4-test ration (originally designed for chicks) and that only slow cures result from administering a highly purified antineuritic vitamin concentrate; also, young rats brought down with polyneuritis by this diet (with the yeast omitted) and cured by crystalline antineuritic vitamin eventually (after 12 to 15 weeks) develop typical B_4 deficiency, curable by adding 2.0 per cent peanuts to their diet; further, young rats fed the polyneuritic diet plus a supplement of 5.0 μg. crystalline B_1 daily from weaning grow slowly, but when B_4 concentrate is also added, they grow five to six times faster.

These results do not necessarily prove the existence of vitamin B_4, inasmuch as each deficiency effect could have been due to deficiency of antineuritic vitamin, and each cure with B_4 concentrate undoubtedly could be duplicated with antineuritic vitamin itself. The evidence still points to the conclusion that in rats, at least, antineuritic vitamin can substitute completely for vitamin B_4.

VITAMIN H.—Boas [241] was the first to observe marked dermatitis and other symptoms in rats fed dried egg white as the sole protein in

[237] Waterman, R. E., and Ammerman, M., Animal Response to Crystalline Vitamine B_1, *J. Biol. Chem.*, 105: *Proc.* xcviii (1934); Studies in Crystalline Vitamin B. V. The Effect of Graduated Doses on Growing Rats, *J. Nutrition*, 10: 35–44 (1935).

[238] Kinnersley, H. W., O'Brien, J. R., and Peters, R. A., Crystalline Vitamin B_1, *Biochem. J.*, 29: 701–715 (1935).

[239] Elvehjem, C. A., and Arnold, A., Interrelationship of Vitamins, *Nature*, 137: 109–110 (1936).

[240] Kline, O. L., Elvehjem, C. A., and Hart, E. B., Further Evidence for the Existence of Vitamin B_4, *Biochem. J.*, 30: 780–783 (1936).

[241] Boas, Margaret A., An Observation on the Value of Egg-White as the Sole Source of Nitrogen for Young Growing Rats, *Biochem. J.*, 18: 422–424 (1924).

an apparently complete ration for growth. Heat-coagulated egg white did not have this effect.[242] Various substitutions in the original diet or additions to it prevented the syndrome,[243] and a protective factor (X) was postulated. Later [244] this hypothesis was abandoned in favor of a direct toxicity effect caused by a substance produced in the drying and destroyed by heat coagulation.

Various vitamins concerned with normal skin development were called H (from *Haut*) vitamins by György.[245] The only "skin" vitamin now called vitamin H is the one preventing the so-called egg-white injury. The most extensive studies on this problem have been made by Parsons [246] and her associates. Salmon and Goodman,[247] Gorter,[248] and Ringrose and Norris [249] have also studied it.

These investigators do not all agree that the phenomenon is to be ascribed to a vitamin deficiency. It is conclusively demonstrated that a prophylactic and curative agent exists in certain food products,

[242] Boas, M. A., A Further Note on the Value of Egg-White as the Sole Source of Nitrogen for Young Growing Rats, *Biochem. J.*, 18: 1322 (1924).

[243] Boas, M. A., The Effect of Desiccation Upon the Nutritive Properties of Egg-White, *Biochem. J.*, 21: 712–724 (1927).

[244] Fixsen, M. A. Boas, The Effect of Desiccation Upon the Nutritive Properties of Egg-White II, *Biochem. J.*, 25: 596–605 (1931).

[245] György, P., *Z. ärztl. Fortb.*, 28: 377 (1931).

[246] Parsons, Helen T., The Physiological Effects of Diets Rich in Egg White, *J. Biol. Chem.*, 90: 351–367 (1931); Further Observations on Egg White Injury in the Rat, *ibid.*, 92: *Proc.* lxiv-lxv (1931); Parsons, H. T., and Kelly, E., The Character of the Dermatitis-Producing Factor in Dietary Egg White as Shown by Certain Chemical Treatments, *ibid.*, 100: 645–652 (1933); The Effect of Heating Egg White on Certain Characteristic Pellagra-like Manifestations Produced in Rats by Its Dietary Use, *Am. J. Physiol.*, 104: 150–164 (1933); Parsons, H. T., Lease, J. G., and Kelly, E., Variations in the Potency of Certain Foodstuffs in the Cure of Dermatitis Induced in Rats by Dietary Egg White, *J. Nutrition*, 8: 57–67 (1934); Lease, J. G., and Parsons, H. T., The Relationship of Dermatitis in Chicks to Lack of Vitamin B_2 and to Dietary Egg White, *Biochem. J.*, 28: 2109–2115 (1934); Parsons, H. T., and Kelly, E., A Comparison of the Antitryptic Activity of Egg White with Its Capacity to Produce a Characteristic Nutritional Disorder, *J. Biol. Chem.*, 116: 685–690 (1936); Parsons, H. T., Lease, J. G., and Kelly, E., The Interrelationship Between Dietary Egg White and the Requirement for a Protective Factor in the Cure of Nutritive Disorder Due to Egg White, *Biochem. J.*, 31: 424–432 (1937); Lease, J. G., Parsons, H. T., and Kelly, E., A Comparison in Five Types of Animals of the Effects of Dietary Egg White and of a Specific Factor Given Orally or Parenterally, *ibid.*, 31: 433-437 (1937).

[247] Salmon, W. D., and Goodman, J. G., Studies of the Raw Egg White Syndrome in Rats, *J. Nutrition*, 8: 1–23 (1934).

[248] Gorter, F. J., Egg-White as Sole Source of Protein and Vitamin B_2 for Young Rats, *Biochem. J.*, 29: 322–329 (1935).

[249] Ringrose, A. T., and Norris, L. C., Differentiation Between Vitamin G and an Insoluble Factor Preventing a Pellagra-like Syndrome in Chicks, *J. Nutrition*, 12: 535–552 (1936); Differentiation Between Vitamin G and a Soluble Factor Preventing a Pellagra-like Syndrome in Chicks, *ibid.*, 12: 553-569 (1936).

notably liver. Parsons and associates believe with Boas-Fixsen that the protective factor is an anti-agent against a positive egg-white injury—a toxicity in a broad sense; Salmon and Goodman also take this view. They admit the possibility of a vitamin deficiency which has been advanced by György, and Ringrose and Norris, but point out that it seems incompatible with the fact that, the greater the concentration of egg white in the ration, the greater the amount of the protective factor necessary for curing the syndrome. Another interesting point is the demonstration that only slight peptic or acid or tryptic digestion or heat denaturation of the harmful egg white renders it "non-toxic." Parsons regards this as a destruction of the toxic principle, Ringrose and Norris as the release or formation of the protective vitamin. It seems surprising that in natural digestion of the harmful egg white similar "destructive" or "synthetic" effects do not occur. A further point is that efforts to concentrate the "toxic" principle have failed, while the protective factor has been not only concentrated but apparently isolated as a definite chemical entity.

Gorter (*loc. cit.*) described some of the solubility properties of the protective factor, and Lease [250] has prepared potent extracts from the papain digest of the insoluble residue left in preparing antianemic principle from liver. Schultz [251] states that vitamin H occurs only in the combined state, from which it must be freed by proteolysis. A 4,000-fold concentration of the vitamin was effected which caused cures in four weeks on subcutaneous injection into rats in 5 μg. daily doses. The concentrate was only one-fifth as effective by mouth. If this may be interpreted as destruction of the vitamin during passage through the digestive tract, it offers a possible explanation of the failure of a protective or vitamin factor released from the undenatured egg white itself during digestion to exert an expected biological effect. Why the result should be different when heat-denatured egg white is fed cannot, as yet, be explained. György [252] describes the same material mentioned by Schultz and its clinical value in severe cases of human seborrhoid dermatitis. György [253] claims that vitamin H is an acidic amino acid.

Vitamin H is required by chicks, rabbits, and monkeys as well as by rats, but probably not by guinea pigs, according to the recent work

[250] Lease, J. G., Some Observations on the Nature and the Partial Isolation of the Substance Curative of the Pellagra-like Condition Due to Dietary Egg-White, *Z. Vitaminforschr.*, 5: 110–118 (1936).

[251] Schultz, F., Vitamin H, *Med. u. chem. Abhandl. med.-chem. Forschungsst., I. G. Farbenind.*, 3: 143–150 (1936).

[252] György, P., Stoffwechsel und Immunbiologie der Haut, *Handb. Kinderheilk.*, Fourth Edition, Vol. 10, Die Hautkrankheit, p. 45, Berlin (1935).

[253] György, P., Attempts to Isolate the Anti-Egg Injury Factor (Vitamin H), *J. Biol. Chem.*, 119: *Proc.*, xliii-xliv (1937).

of Parsons and associates. Chicks are more sensitive to the egg-white injury than are rats.

VITAMIN K.—Dam,[254] and McFarlane and associates[255] observed fatal hemorrhagic disease in young chicks after several weeks of feeding synthetic diets, outstanding symptoms being intestinal, subcutaneous, and intramuscular hemorrhage, inability of the blood to clot, and erosion of the gizzard lining. Dam was not successful in preventing this with lemon juice. Holst and Halbrook[256] reported the same disease occurring on a natural ration; they found it curable by small amounts of cabbage. Shortly after this, Dam[257] returned to a study of the disease. All the evidence pointed to a deficiency disease, but since no relation was found to deficiencies in vitamins, A, B_1, B_2, C, D, E, fat or cholesterol, Dam proposed that the antihemorrhagic factor be called vitamin K (Koagulations-Vitamin).

Present knowledge regarding the chemistry of vitamin K is the result of the efforts of Dam and associates,[258] and of Almquist,[259] to isolate it. The vitamin appears to be a colorless, crystalline, optically inactive, low-melting, waxy, unsaturated compound, containing a benzene ring or rings, but free from N, S, and P, $-\overset{|}{C}=O$, or $-\overset{|}{C}OH$. It is highly soluble in polar and non-polar organic solvents and should be classed with the fat-soluble vitamins. It sublimes at 160° C., 0.01 mm. It shows strong general absorption of the ultraviolet but no characteristic bands. Ultraviolet rays will destroy its activity, but in general it is stable to heat and light. It is very unstable to alcoholic alkalies and acetic anhydride. Halogenation renders it inactive. It is readily adsorbed by activated carbon, Al_2O_3, MgO, $MgCO_3$, $CaCO_3$, sucrose, and fuller's earth, but these adsorption products are not bio-

[254] Dam, H., Cholesterinstoffwechsel in Hühnereiern und Hühnchen, *Biochem. Z.*, 215: 475–492 (1929); Über die Cholesterinsynthese im Tierkörper, *ibid.*, 220: 158–163 (1930).

[255] McFarlane, W. D., Graham, W. R., Jr., and Richardson, F., The Fat-Soluble Vitamin Requirements of the Chick. I. The Vitamin A and Vitamin D Content of Fish Meal and Meat Meal, *Biochem. J.*, 25: 358–366 (1931).

[256] Holst, W. F., and Halbrook, E. R., A "Scurvy-Like" Disease in Chicks, *Science*, 77: 354 (1933).

[257] Dam, H., Hemorrhages in Chicks Reared on Artificial Diets. New Deficiency Disease, *Nature*, 133: 909–910 (1934); Dam, H., and Schönheyder, F., A Deficiency Disease in Chicks Resembling Scurvy, *Biochem. J.*, 28: 1355–1359 (1934); Dam, H., The Antihaemorrhagic Vitamin of the Chick, *Nature*, 135: 652–653 (1935); *Biochem. J.*, 29: 1273–1285 (1935).

[258] Dam, H., and Schönheyder, F., The Occurrence and Chemical Nature of Vitamin K, *Biochem. J.*, 30: 897–901 (1936); Dam, H., and Lewis, L., The Chemical Concentration of Vitamin K., *ibid.*, 31: 17–21 (1937).

[259] Almquist, H. J., Purification of the Antihemorrhagic Vitamin, *J. Biol. Chem.*, 114: 241–245 (1936); Chemical and Physical Studies on the Antihemorrhagic Vitamin, *ibid.*, 117: 517–523 (1937); Further Studies on the Antihemorrhagic Vitamin, *ibid.*, 120: 635–640 (1937).

logically active; they are useful in effecting concentration of the vitamin.

Schönheyder [260] has developed a biological assay procedure for vitamin K based on its ability to restore the clotting of the blood which is inhibited in severe vitamin K deficiency. One unit of the vitamin is the smallest quantity which can be administered during a certain time (several days) to sick chickens having a specified degree of the hemorrhagic disease, in order to reduce the clotting time to an arbitrary normal. This procedure is criticized by Almquist and Stokstad,[261] who have developed a method based on prevention of prolonged clotting time and hemorrhage in chicks after being on the vitamin K-free diet for two weeks after hatching. This procedure gives no numerical values except percentage levels of foods which protect birds from the disease.

Either procedure apparently shows that all green plant material is rich in the vitamin, artificially dried alfalfa being used as the chief source of the vitamin for chemical studies. Hog-liver fat is another rich source. Hempseed was found by Dam to be a good source, also certain vegetables, but cereals and other seeds in general are poor sources. Almquist and Stokstad [262] found that the vitamin is produced, presumably by microorganisms, in fish meal and rice bran when they are allowed to remain in a wet condition for several days at room temperature. Spontaneous cures may occur, possibly explainable by coprophagy. Egg yolk fat and soybean oil were found to contain the vitamin.

According to Dam and associates,[263] vitamin K deficiency can be produced in young ducklings and geese, and very mildly in pigeons and canaries, but not in rats, guinea pigs, and dogs, or in man. However, Butt, Snell, and Osterberg [263a] have shown the vitamin to be useful in the treatment of hemorrhage in obstructive human jaundice, suggesting its normal need by man. It is not known whether vitamin

[260] Schönheyder, F., Antihemorrhagic Vitamin of the Chick—Measurement and Biological Action, *Nature*, 135: 653 (1935); The Determination of Vitamin K, *Biochem. J.*, 30: 890–896 (1936).

[261] Almquist, H. J., and Stokstad, E. L. R., Assay Procedure for Vitamin K (Antihemorrhagic Vitamin), *J. Nutrition*, 14: 235–240 (1937).

[262] Almquist, H. J., and Stokstad, E. L. R., Dietary Haemorrhagic Disease in Chicks, *Nature*, 136: 31 (1935); Hemorrhagic Chick Disease of Dietary Origin, *J. Biol. Chem.*, 111: 105–113 (1935); Factors Influencing the Incidence of Dietary Hemorrhagic Disease in Chicks, *J. Nutrition*, 12: 329–335 (1936).

[263] Dam, H., Schönheyder, F., and Lewis, L., The Requirement for Vitamin K of Some Different Species of Animals, *Biochem. J.*, 31: 22–27 (1937).

[263a] Butt, H. R., Snell, A. M., and Osterberg, A. E., The Use of Vitamin K and Bile in Treatment of the Hemorrhagic Diathesis in Cases of Jaundice, *Proc. Staff Meetings Mayo Clinic*, 13: 74–77 (1938); *cf.* also Osterberg, A. E., Vitamin K: Its Distribution and Chemical Properties; Methods of Preparation and Assay, *ibid.*, 13: 72–74 (1938); *cf. Proc. Soc. Exptl. Biol. Med.*, 38: 336–338 (1938).

K deficiency occurs in rabbits and other herbivora, although Quick [264] has pointed out its possible analogy to sweet clover disease and a possible relation to vitamin K of the curative agent for this disease in alfalfa.

CHICK GIZZARD FACTOR.—The early work leading to the discovery of vitamin K pointed out the gizzard erosions which accompanied the hemorrhagic syndrome. This is still regarded by Dam [265] as one of the manifestations of vitamin K deficiency. On the other hand, Almquist and Stokstad [266] regard the gizzard defect as caused by a distinct deficiency. This view is supported by Bird and associates.[267] Almquist [268] has made further studies of the chemical properties of the factor which has not yet been given specific vitamin designation.

Evidence for the existence of this factor distinct from vitamin K rests on the findings that the anti-gizzard-erosion factor is not present in highly concentrated preparations of vitamin K, is a saponifiable compound (appears in soap fractions), and occurs in the cereal grains and other products which lack the antihemorrhagic vitamin. In further contrast to vitamin K it is unstable to heat. Almquist found that normal gizzard linings contain the factor.

CITRIN (VITAMIN P).—In 1936 a group of Hungarian workers, including Szent-Györgyi,[269] reported cures of purpura hemorrhagica by administering lemon juice but not when giving ascorbic acid. A flavone glycoside was isolated which cured vascular forms of purpura. This was named citrin (citrus flavone) to indicate its chemistry and also vitamin P to indicate its vitamin-like effect on tissue permeability (*i.e.*, Permeabilität Vitamin). The citrin also prolonged the life of scorbutic guinea pigs and reduced the extent of capillary hemorrhages.

[264] Quick, A. J., The Coagulation Defect in Sweet Clover Disease and in the Hemorrhagic Disease of Dietary Origin. A Consideration of the Sources of Prothrombin, *Am. J. Physiol.*, 118: 260-271 (1937).

[265] Dam, H., Schönheyder, F., and Lewis, L., The Requirement for Vitamin K of Some Different Species of Animals, *Biochem. J.*, 31: 22–27 (1937).

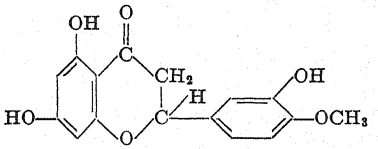
[266] Almquist, H. J., and Stokstad, E. L. R., Hemorrhagic Chick Disease of Dietary Origin, *J. Biol. Chem.*, 111: 105-113 (1935); Nutritional Deficiency Causing Gizzard Erosions in Chicks, *Nature*, 137: 581–582 (1936); The Gizzard Factor of the Chick, *J. Nutrition*, 13: 339–350 (1937).

[267] Bird, H. R., Kline, O. L., Elvehjem, C. A., Hart, E. B., and Halpin, J. G., The Distribution and Properties of the Anti-Gizzard-Erosion Factor Required by Chicks, *J. Nutrition*, 12: 571–582 (1936).

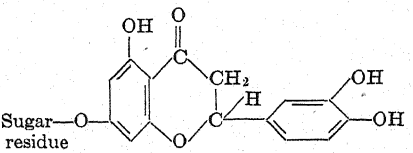
[268] Almquist, H. J., Sources and Nature of the Chick Gizzard Factor, *J. Nutrition*, 14: 241–245 (1937).

[269] Rusznyák, I., and Szent-Györgyi, A., Vitamin P: Flavonols as Vitamins, *Nature*, 138: 27 (1936); Armentano, L., Bentsáth, A., Beres, T., Rusznyák, I., and Szent-Györgyi, A., Über den Einfluss von Substanzen der Flavongruppe auf die Permeabilität der Kapillaren, Vitamin P. *Deut. med. Wochschr.*, 62: 1326–1328 (1936); Bentsáth, A., Rusznyák, St., and Szent-Györgyi, A., Vitamin Nature of Flavones, *Nature*, 138: 798 (1936).

Chemical studies [270] of citrin showed it to be a mixture of previously known flavones, namely hesperidin and eriodictyol, the latter being present as a glycoside, from which it could be freed by hydrolysis. Hesperidin is a methyl derivative of eriodictyol.



Hesperidin



Eriodictyol glycoside

Bruchner and Szent-Györgyi (*loc. cit.*) regarded the eriodictyol glycoside as the more active functional part of citrin. Zilva found it to comprise one-third of the mixed flavone. The Hungarian workers [271] have concluded that experimental scurvy is the result of combined ascorbic acid and citrin avitaminoses, pure ascorbic acid deficiency being obtained only when vitamin P is supplied. This is denied by Zilva (*loc. cit.*). The difference between the two series of observations can be reconciled, according to Bentsáth and Szent-Györgyi,[272] by assuming that vitamin P requires traces of ascorbic acid for its activity.

FACTOR W.—Elvehjem, Koehn, and Oleson [273] describe complete failure of young rats to grow when fed a synthetic ration in which all the known vitamins needed by rats (A, B_1, D, E, riboflavin, B_4, B_6, and "P.P." factor) were supplied in the supplements. Growth was restored by liver, yeast, and milk. No specific pathology accompanied the growth failure. Evidence was obtained of a water-soluble, heat-labile factor, the deficiency of which is regarded as responsible for the growth failure. The factor is tentatively called [274] factor W. Concentrates have been prepared from liver which are effective in restoring growth of 3.5 to 4.0 grams daily in less than 1.0 mg. dry matter daily doses. Kidney tissue is also rich in the factor, but wheat germ, rice bran, and cereal grains are low. The factor is adsorbed by charcoal but not by fuller's earth. It is stable to ultraviolet rays.

[270] Bruckner, V., and Szent-Györgyi, A., Chemical Nature of Citrin, *Nature*, 138: 1057 (1936); Lajos, S., and Gerendás, M., Spektrographische Untersuchung des Vitamins "P" (Citrin) und anderer flavonartiger Stoffe, *Biochem. Z.*, 291: 229–236 (1937); Zilva, S. S., Vitamin P, *Biochem. J.*, 31: 915–919 (1937).

[271] Bentsáth, A., Rusznyák, I., and Szent-Györgyi, A., Vitamin P., *Nature*, 139: 326–327 (1937).

[272] Bensáth, A., and Szent-Györgyi, A., Vitamin P., *Nature*, 140: 426 (1937).

[273] Elvehjem, C. A., Koehn, C. J., Jr., and Oleson, J. J., A New Essential Dietary Factor, *J. Biol. Chem.*, 115: 707–719 (1936).

[274] Frost, D. V., and Elvehjem, C. A., Further Studies on Factor W, *J. Biol. Chem.*, 119: *Proc.* xxxiv-xxxv (1937); *ibid.*, 121: 255-273 (1937).

CHAPTER XXXVIII

ENZYMES

CATALYSIS.—It has long been known that the velocity of certain reactions can be increased by the presence of relatively small amounts of certain substances. For example, hydrogen and oxygen gases do not combine at a measurable speed at ordinary temperature, but when a mixture of such gases is passed over platinum black, combination takes place, the reaction proceeding at such a velocity that the platinum black may actually become incandescent. A similar reaction is used for the synthesis of sulfur trioxide in the manufacture of sulfuric acid and is known as the contact process. Finely divided platinum has the property of greatly speeding up the decomposition of hydrogen peroxide.

Substances which have this remarkable power of hastening a chemical reaction are called catalytic agents, and their action is known as catalysis, from the Greek κατα meaning "down," and λυο meaning "loosen," literally a down-loosening, which has come to mean the hastening of a chemical reaction.

Until rather recently it was generally agreed that there were two properties common to all catalysts, (1) that the catalyst did not initiate a new reaction but merely changed the rate of a reaction already in progress, and (2) that a catalyst did not appear in the final products of the reaction which was accelerated and that it was not used up in the process of the reaction. There has been a revision of opinion in regard to the first hypothesis, *i.e.*, that a catalyst is incapable of initiating a new reaction. According to the old belief the explanation for the speeding up of the reaction between the gases, hydrogen and oxygen, was that the reaction proceeded at room temperature, but the rate of the reaction was essentially negligible and could not be detected within measurable time. The newer conception is that *a catalyst may actually initiate a chemical reaction.* This viewpoint is excellently summed up by Reid [1] in the statement, "The old conception according to which the catalyst contributes only its presence and takes no part in the reaction has been given up. Zelinsky [2] remarks:

[1] Reid, E. E., Fifth Report of the Committee on Contact Catalysis, *J. Phys. Chem.*, 31: 1121–1149 (1927).

[2] Zelinsky, N. D., Über die Inaktivierung der Katalysatoren bei den Umwandlungen von Kohlenstoffverbindungen, *Ber.*, 59: 156–162 (1926).

'My observations on catalysts extending over several years have brought me to the same view of catalytic phenomena as was expressed by Mendelejeff [3] long ago in such a simple and original form, a view which later Raschig [4] and recently it seems Bodenstein [5] have adopted. In the contact processes with carbon compounds, the catalyst does not determine the reaction simply by its presence but by taking the role of an active principle in the process; its surface energy produces far-reaching alterations in the substances which come into contact with it.' "

Boswell and Dilworth,[6] in discussing the mechanism of catalysis by aluminum oxide, go a step further, and after discussing the nature of the surface film surrounding the aluminum oxide particle, state, "This all points to the conclusion that this catalyst functions by means of a surface film of water and that this film is the real seat of the catalysis." Their conception is an *orientated adsorption.* After further discussing certain specific reactions catalyzed by aluminum oxide, Boswell and Dilworth continue, "The catalyst from this point of view does not accelerate a reaction already in progress . . . but actually initiates the change . . ." Also, there is now abundant proof that in many instances the catalyst does enter into the reaction—at least to the extent of forming labile compounds with the reactive molecules of the substrate. These labile compounds then are rearranged or altered while associated with the catalyst and break down with the formation of new products and the regeneration of the catalyst.

These modifications of the older conception are of extreme importance and afford a logical explanation for the behavior of a catalyst. According to the older viewpoint the catalyst was looked upon as a mysterious chemical compound which in some way speeded up a reaction. According to the newer viewpoint a catalyst is looked upon as a source of *surface energy*, the chemical nature of the catalyst being relatively unimportant providing that the space configuration of the atoms in the surface of the catalyst are such as to cause certain oriented adsorption relationships and the surface of the catalyst is in such a state as to contribute a given quantity of surface energy to the system. This surface energy is probably effective by inducing electron displacements in the adsorbed and oriented molecules so that they become "activated" or "ionized" and thus more chemically reac-

[3] Mendelejeff, D., *J. Russ. Chem. Soc.*, 18: 8–18 (1886) (in Russian) *cf.* also Notiz über Contactwirkungen, *Ber.*, 19: 456–463 (1886).

[4] Raschig, F., Gedanken über Katalyse, *Z. angew. Chem.*, 19: 1748–1763 (1906). *Cf.* also Bredig, G., Bemerkungen zu Raschig's "Gedanken über Katalyse," *Z. angew. Chem.*, 19: 1984–1987 (1906).

[5] Bodenstein, M., Ein Beitrag zur Theorie der katalytischen Hydrierung durch Platin, *Ann.*, 440: 177–185 (1924).

[6] Boswell, M. C., and Dilworth, H. M., On the Mechanism of Catalysis by Aluminum Oxide, *J. Phys. Chem.*, 29: 1489–1506 (1925).

tive. The shift of an electron bond may thus cause an intramolecular rearrangement, or two or more "activated" molecules may react to form new compounds or polymers, or two unlike molecules may react to form new compounds with more stable electron arrangements. It has been generally recognized that many substances of the most diverse chemical nature may be used to catalyze a given reaction. The difficulty in the past has been to explain such catalysis on the basis of the diverse chemical nature of the catalysts which were involved. Under the new viewpoint where only surface energy forces and oriented adsorption are regarded as important, it is very easy to see how many diverse substances in the proper physical state, *particularly so far as the spatial relationships of the surface atoms are concerned,*[7] may induce identical electron shifts in the molecules which are adsorbed and actually accelerate reactions at more or less the same rate. Similarly this viewpoint affords a logical explanation of the newer belief that a catalyst may initiate a reaction which is not already in progress by inducing electron shifts in the reacting molecules, which shifts are necessary for the initiation.

A catalyst, therefore, must be looked upon as a substance which alters the rate of reaction, but it may be either in the direction of acceleration or of retardation. The reaction may be one that in the absence of a catalyst proceeds relatively rapidly or proceeds so slowly that it requires special proof to show that it is taking place at all, or at least in certain instances is not, in the absence of the catalyst, detectable by any means as yet at the disposal of the chemist.

Catalysts do not appear, as a rule, in the end products of the reaction, but there is no doubt that in many cases, if not in all, the catalytic surface has actually entered into the reaction at some stage or other, or at least that an oriented adsorption has taken place upon the surface of the catalyst and in this way the reacting molecules have been brought within the sphere of chemical attraction and chemical reactivity. If the products of the reaction are less strongly adsorbed upon the catalyst than are the reacting materials, the reaction products will leave the surface of the catalyst, allowing additional adsorption of the reacting materials, which in turn will combine, thus speeding up the rate of formation of the final products.

In the discussion of adsorption and molecular orientation we considered Quastel's theory of "active centers" on the bacterial surface mosaic. Certain compounds were adsorbed on specific centers. A part of these compounds were "activated" and reacted as hydrogen

[7] Berkman, S., Morrell, J. C., and Egloff, G., The Phenomenon of Catalysis, Booklet 207, Universal Oil Products Company, Chicago (1936); *cf.* also Inhibitors in Catalysis, Booklet 206, Universal Oil Products Company, Chicago (1936), and Promoters and Poisons in Catalysis, Booklet 208, Universal Oil Products Company, Chicago (1936).

donators. Other compounds, while they were adsorbed, were not activated. These remained on the active surface and blocked the adsorption and activation of the reactive molecules. These compounds which were adsorbed but were not activated were thus "catalyst poisons." They were "sit-down strikers" who filled the factory and not only did no work themselves but prevented others from working. This represents one type of catalyst inhibition. Another type is those inhibitors which break the chain in chain reactions. We have noted that the inhibitols in the natural fats and oils act by breaking chains formed in chemical reactions, and Alyea and Pace [8] have shown that the decomposition of hydrogen peroxide by catalase is a chain reaction, with the chain starting at the surface of the enzyme and extending outward for at least 2,000 molecules, and that inhibitors of catalase action act, not on the enzyme itself, but rather by breaking the chain almost as soon as it is started.

In some instances the role of a catalyst is to accept energy evolved by one chemical reaction and to transfer that energy so that it can be used in another chemical reaction. Such systems of reactions are known as "coupled reactions." Schott and Borsook [9] found that toluene-treated cells of *B. coli.* exhibited two coupled reactions, (1) the reduction of pyruvate to lactate by means of the energy liberated by the anaerobic oxidation of formate to bicarbonate, and (2) the reduction of fumarate to succinate through the anaerobic oxidation of lactate to pyruvate. In each instance an intermediate "carrier" or catalyst is necessary. Methylene violet served as the carrier for the first system and methylene blue as the carrier for the second system. *The energy-liberating and energy-absorbing mechanisms are separate.* The carrier transports the energy from where it is liberated to where it is absorbed. *No reaction occurs without the carrier. The half reaction does not take place.* The authors note that these observations support Quastel's hypothesis of active centers on the bacterial mosaic.

Although the degree of acceleration of a reaction is, within certain limits, proportional to the concentration of the catalyst present, the final equilibrium appears to be independent of the amount of catalyst. It is surprising how minute a quantity of a catalyst is capable of perceptible catalytic activity. For example, colloidal platinum will decompose a million times its weight of hydrogen peroxide. Since the addition of a catalyst increases the rate of a chemical reaction, it follows that the time required to produce a definite equilibrium must vary with the amount of catalyst which is present.

Water is one of the most important catalytic agents which are

[8] Alyea, H. N., and Pace, J., Inhibitors in the Decomposition of Hydrogen Peroxide by Catalase, *J. Am. Chem. Soc.*, 55: 4801–4806 (1933).

[9] Schott, H. F., and Borsook, H., Coupled Reactions in Biological Systems, *Science*, 77: 589 (1933).

known. In certain instances the presence of water accelerates a reaction, in other instances retards or inhibits a reaction, especially when water is a product of the reaction. Perfectly dry ammonia and hydrogen chloride gases will not combine with a measurable speed, but in the presence of traces of water the reaction is almost instantaneous. Chlorine and hydrogen gases when pure will not combine at measurable speed to form hydrogen chloride, but in the presence of traces of water the reaction is greatly accelerated. As we shall see later, water enters into many of the reactions characteristic of the biocatalysts.

ENZYMES AS CATALYSTS.—The catalytic agents of cells and of the constituents of living organisms are known as enzymes, a word derived from the Greek, meaning literally "in yeast" or "in leaven." An enzyme thus may be regarded as an organic catalytic agent found in living matter. These catalytic agents are very numerous, and it is to them that we ascribe the chemical reactions which occur in living protoplasm. The food of plants and of animals, the proteins, carbohydrates, fats, etc., is in general valueless unless it is brought into a condition suitable for assimilation and translocation. The starch of the leaf must be rendered soluble before it can be transported to other parts of the plant. The starch of the potato tuber must similarly be rendered soluble before it can be used for the nutrition of the young shoots. The starch of our foods must be rendered soluble before it will pass from the intestinal tract into the blood stream and from the blood stream to the tissues of the body. The glycogen which is stored in the liver as a reserve source of energy must similarly be rendered soluble by the action of enzymes before it can be utilized as a source of energy by the organism. Similarly the proteins, the fats, the lipids, and, in general, all the energy- and tissue-building constituents of both plants and animals must be acted upon by one or more enzymes before they are rendered available for the use of the organism. Thus, it is seen that enzyme action is a strategic center of vital activity.

In 1811, Kirchhoff [10] noted that starch was transformed by mineral acids into glucose, and he demonstrated that no acid was consumed in the process. This then is an example of a chemical reaction catalyzed by hydrogen ions. In 1833, Payen and Persoz [11] made the discovery which has had far-reaching consequences, *i.e.*, that germinating seeds contain a peculiar contact substance which transforms starch into sugar. This substance they called *diastase*, a name which still persists in the literature, although *amylase* appears preferable. Inasmuch as diastase is concerned with the hydrolysis of starch and is the active

[10] Kirchhoff, G. S., Ueber die neue von Kirchhoff entdeckte Zuckergewinnung, *Schweigger's J. Chem. Phys.*, 4: 108–110 (1811).

[11] Payen and Persoz, Mémoire sur la diastase. Les principaux produits de ses reactions, et leurs applications aux arts industriels, *Ann. chim. phys.*, 53: 73–92 (1833).

principle in malt, diastase has probably been studied more extensively than any other enzyme. Therefore, the real beginning of enzyme study dates back to the observations of Payen and Persoz.

It is known that a great number of chemical reactions are what are termed reversible reactions. An example of such a reaction is the formation of acetic acid and alcohol from ethyl acetate and water:

$$\text{Ethyl acetate} + \text{water} \rightleftharpoons \text{ethyl alcohol} + \text{acetic acid}$$

The characteristic of a reaction of this kind is that it progresses in one direction or the other until a certain equilibrium specific to the reaction and to the concentration of the reacting materials is established. Any alteration in concentration of the reacting substances is immediately followed by a change to a new equilibrium. The velocity of the reaction moreover may be accelerated by the presence of either hydrogen or hydroxyl ions which, although they do not affect the nature of the final products, accelerate the process and alter the position of equilibrium. In this instance, either hydrogen or hydroxyl ions may be regarded as catalysts.

In the plant or in the animal organism many reactions are known to be of the above type, as for instance the hydrolysis of starch into sugar and the synthesis of starch again from the sugar. Outside of the living organism the hydrolysis of starch by purely chemical processes takes place only at an elevated temperature or in the presence of an appreciable concentration of hydrogen ions. Within the living organism or in the presence of enzymes the hydrolysis occurs at ordinary temperatures with considerable rapidity and is controlled by the amyloclastic enzymes. Amylase, therefore, is a catalyst produced by the living organism. The extraction of amylase and the demonstration of its catalytic action *in vitro* is, of course, a perfectly simple experiment, but the synthesis of starch from sugar by the same enzyme has not as yet been carried out *in vitro*. It is reasonable to assume, however, that amylase controls the process in both directions, and there are other instances where the synthetic power of enzymes has been demonstrated *in vitro* under special conditions; for example, the synthesis of glucosides by emulsin, the synthesis of salmin from its split-products by trypsin, and the synthesis of plasteins from proteoses and peptones by the action of pepsin have already been noted. Sym [12] has described conditions under which the synthesis of specific esters by lipases and esterases is greatly accelerated. He discovered that the sodium salts of the bile acids and the sodium soaps of the fatty acids very strongly activate the enzymatic ester synthesis. The synthesis is carried out in a benzene or carbon tetrachloride medium which serves as a good solvent for the esters which

[12] Sym, E. A., Eine Methode der enzymatischen Estersynthesen, *Enzymologia*, 1: 156–160 (1936).

are formed. Pancreatin is added to this medium together with the fatty acid and the alcohol which it is desired to esterify. An aqueous 30 per cent solution of bile salts is then added and the mixture shaken in a water thermostat at 37°. In the case of benzoic acid-butyl alcohol almost complete esterification resulted at the end of 48 hours. Cholesterol-butyric acid ester was similarly formed in excellent yield under similar conditions. While other workers have shown the reversibility of lipase and esterase activity, the technic of Sym provides a method whereby the desired esters can be synthesized in rather large amounts. Although the reversibility of the action of a number of enzymes has been demonstrated *in vitro,* it is not necessary to postulate that all enzyme reactions are reversible reactions. It is entirely probable that in some instances one enzyme may control the synthetic process, and another, and a different enzyme, the process whereby the substrate is broken down.

FERMENTATION.—The formation of alcohol and carbon dioxide from sugar, on account of the effervesence or apparent boiling due to the escape of carbon dioxide, was early called fermentation from the Latin, *fervere,* to boil. When Pasteur showed that this process was due to the presence of a living organism, diastase and other biocatalysts analogous to diastase were called "soluble or unorganized ferments" to distinguish them from living organisms, such as yeasts and certain bacteria, which were termed "organized ferments." On account of the confusion of the terms it was suggested by Kühne, in 1878, that the "soluble ferment" should be termed "enzyme," which at that time signified that something in yeast which caused the fermentation of sugar.

It was definitely shown by Buchner[13] that yeast did contain an enzyme which fermented sugar in the absence of the living cells. The yeast was ground so that the contents of the cells could be extracted by pressure. The liquid so obtained, freed by filtration from every trace of living cells or their fragments, still possessed the property of causing alcoholic fermentation analogous to, although at a lower rate than, that caused by the living yeast. He also demonstrated that the alcohol-forming substance was soluble in water, precipitable by alcohol, and very easily destroyed by heat.

DEFINITION OF ENZYMES.—Waldschmidt-Leitz[14] defines enzymes as *"definite material catalyzers of organic nature with specific powers of reaction, formed indeed by living cells, but independent of the presence of the latter in their operation."* This definition appears to be fairly satisfactory, although it should probably have appended to

[13] Buchner, E., Alkoholische Gärung ohne Hefezellen, Vorlauf, Mitt., *Ber.,* 30: 117–124 (1897); Zweite Mitt., *Ber.,* 30: 1110–1113 (1897).

[14] Waldschmidt-Leitz, E., Enzyme Actions and Properties, translated and extended by R. P. Walton, John Wiley & Sons, New York (1929).

it the phrase, *and, when in the moist state, readily destroyed by heat.*

Occurrence of Enzymes.—Enzymes may occur in the secretions of living organisms, and as such act outside of the normal protoplasmic mass. Such enzymes are known as extracellular or secretion enzymes, and in the living organism occur in secretions which appear to pass through the living cell membrane. Such enzymes normally act outside of the cells which produce them. Ptyalin, the amylase of the saliva, pepsin, the protease of the gastric juice, and sucrase, the inverting enzyme of yeast, are typical examples of extracellular enzymes. Preparations of such enzymes are rather readily obtained in quantity, and most of our knowledge of enzymatic behavior has been gained through a study of the behavior of extracellular enzymes.

The intracellular enzymes appear to be non-diffusible through the cell membrane. They are not excreted by the living organism but instead act inside of the cell. In some instances it is possible to destroy the protoplasm without inactivating the enzymes. In other instances it has not as yet been possible to prepare enzyme preparations which reproduce *in vitro* the chemical reactions characteristic of living organisms and which reactions we have every reason to believe are due to the presence of intracellular enzymes.

Three general methods are available for the preparation of intracellular enzymes. Buchner proposed the method of grinding the cellular material with sand and fuller's earth and placing this ground mass in a hydraulic press, certain of the intracellular enzymes being released by the grinding process and appearing in the press liquor. In this way he demonstrated the presence of zymase as the intracellular fermenting enzyme of yeast. It should be noted, however, that this method permits only the demonstration of intracellular enzymes which are soluble in water or which disperse in water to form colloidal sols. Any enzymes which may be insoluble in water or which are strongly adsorbed upon the cell wall fragments or upon the sand or fuller's earth surfaces would not appear in the extract.

Rowland modified the method of Buchner in that he froze the cellular mass and then ground the frozen material in a mill. Wiechowski later modified this method by pulping the tissue, pressing it through a sieve, drying it at a low temperature, and then grinding the dried material with toluene in a paint mill. The fine powder was removed from the toluene suspension and dried. All cells had been disintegrated by this method, and the dry cell-free powder was used as an enzyme preparation.

The Classification of Enzymes.—We have already noted many instances of enzyme action in connection with the proteins, carbohydrates, glycosides, fats, lipids, and pigments, all of which serve as substrates and are altered by the action of one or more enzymes. Accord-

ingly in this chapter we will not include a discussion of the specific enzymes in relation to specific reactions. The enzyme literature is exceedingly voluminous, as is evidenced by the compilations of Oppenheimer [15] and Euler,[16] and the fact that even though two journals, *Fermentforschung* and *Enzymologia,* are devoted exclusively to contributions in this field, nevertheless a very considerable fraction of the papers appearing in other biochemical journals deal with enzymatic studies.

The great majority of enzymes are those which may be classed as hydrolyzing enzymes, indicating that the elements of water enter into the chemical reaction and that the reactions usually take place in the presence of an excess of water. This factor is sometimes overlooked as an important factor in enzyme phenomena, whereas in reality the relative abundance of water is probably an extremely important factor in governing the direction and position of the final equilibrium.

In the following classification have been listed most of the enzymes for which adequate characterizations have been given, together with the substrate upon which the enzyme acts and the end products of the reaction in so far as the end products are actually known. It is entirely probable that certain of the enzymes which, with our present knowledge, have been given specific names may in reality be composed of a mixture of enzymes. Thus, pancreatic trypsin has been shown [17] to be a mixture of at least three enzymes, (*a*) crystalline trypsin, (*b*) chymotrypsin, and (*c*) heterotrypsin. These are distinguished by their action on three different substrates. Benzoylarginineamide is hydrolyzed by crystalline trypsin but not by the other two enzymes. Carbobenzoxytyrosylglycineamide is hydrolyzed by chymotrypsin and not by the other two enzymes. Benzoylglycyllysineamide is hydrolyzed by heterotrypsin and not by either chymotrypsin or crystalline trypsin. Similarly erepsin from the intestinal juice of swine contains [18] at least two polypeptidases. One has an optimum (under the experimental conditions) at *p*H 7.3 and hydrolyzes glycylglycine and leucylglycine at the same rate. The other under similar conditions has an optimum at *p*H 8.1 and hydrolyzes leucylglycine appreciably faster than it does glycylglycine. Fractional adsorption on aluminum hydroxide serves to partially separate these

[15] Oppenheimer, C., Die Fermente und ihre Wirkungen, Fifth Edition, 4 Vols., Georg Thieme, Leipzig (1925–1929); Supplement Bd., W. Junk, The Hague (1936–1937).

[16] Euler, Hans von., Chemie der Enzyme, Third Edition, 2 Vols., J. F. Bergmann, Munich (1925–1934).

[17] Bergmann, M., Fruton, J. S., and Pollok, H., The Differentiation of Pancreatic Trypsins on the Basis of Their Specificities, *Science,* 85: 410–411 (1937).

[18] Linderstrøm-Lang, K., Über Darmerepsin, *Z. physiol. Chem.,* 182: 151–174 (1929).

enzymes. While some of the named enzymes unquestionably represent mixtures, it is still more certain that other unknown enzymes, which have not been identified and studied, must exist, so that the following grouping must be regarded as one which will have to be altered from time to time as additional information is obtained.

CLASSIFICATION OF ENZYMES

	Substrates	End Products
A. Hydrolyzing enzymes		
I. Esterases		
1. Lipases		
a. glyceridases	Glycerides	Glycerol + fatty acids
b. Others (uncertain)	Fatty acid esters of alcohols other than glycerol	Alcohol + fatty acids
2. Choline esterase	Choline esters (especially acetylcholine)	Choline + acetic acid
3. Chlorophyllase	Chlorophyll-*a*	Chlorophyllide-*a* + phytol
4. Pectase	Pectin	Pectic acid + MeOH
5. Cholesterase	Cholesterol esters	Cholesterol + R COOH
6. Tannase	Tannin	Glucose + gallic acid
7. Phosphatases		
a. Lecithinase	Lecithin	Choline + H_3PO_4 + fat
b. Lecithinase A	Lecithin	Lysolecithin + unsat. fatty acid
c. Phosphodiesterase	Diesters of orthophosphoric acid	Alcohol + monoester
d. Phosphomonoesterase	Monoesters of orthophosphoric acid	Alcohol + phosphoric acid
e. Hexosediphosphatase	Hexosediphosphoric acid	Hexose + H_3PO_4
f. Phosphatese (synthesizing)	Hexose + H_3PO_4	Hexosephosphate
g. Polynucleotidase	Nucleic acid	Mono-nucleotides
h. Yeast polynucleotidase	Yeast nucleic acid	Mono-nucleotides
i. Phosphonuclease	Nucleic acid	Mono-nucleosides + H_3PO_4
j. Phytase	Phytin	Inositol + H_3PO_4
8. Sulfatase	Phenol sulfates	Phenol + $KHSO_4$
II. Carbohydrases		
1. Fructosidases (sucrase)	(a) Sucrose	Fructose + glucose
	(b) Raffinose	Fructose + melibiose
	(c) Gentianose	Fructose + gentiobiose
	(d) Stachyose	Fructose + mannotrisaccharide
2. α-Glucosidases		
a. Maltase	Maltose	Glucose
b. Trehalase	Trehalose	Glucose
c. Others (uncertain)	α-Glucosides	Glucose + non-sugar

CLASSIFICATION OF ENZYMES—*Continued*

	Substrates	End Products
3. β-Glucosidases		
a. Emulsin (mixture)	All β-Glucosides	Sugar + non-sugar residue
b. Amygdalase	Amygdalin	Glucose + prunasin
c. Prunase	Prunasin	Glucose + *d*-mandelonitrile
d. Cellobiase	Cellobiose	Glucose
e. Gentiobiase	Gentiobiose	Glucose
4. Other glucosidases		
a. Oxynitrilase	Oxynitrile	Aldehyde + HCN
b. Vicianase	Vicianin	Benzaldehyde + HCN + glucoarabinose
c. Robininase (rhamnodiastase)	Robinine	Camphor + robinose
5. β-Galactosidases		
a. Lactase	Lactose	Galactose + glucose
b. Melibiase	Melibiose	Galactose + glucose
6. Amylase (liquefying)	Insoluble starch	Soluble starch
7. α-Amylase (saccharifying)	Soluble starch	α-Maltose
8. β-Amylase (saccharifying)	Soluble starch	β-Maltose
9. Cellulase	Cellulose	Cellobiose
10. Hemicellulases (cytase)	Hemicelluloses	Simple sugars
11. Lichenase	Lichenin	Cellobiose
12. Inulase	Inulin	Fructose
13. Protopectinase	Protopectin	Pectin
14. Pectinase	Pectic acid	Galactose + galacturonic acid
III. Enzymes hydrolyzing nitrogen compounds		
1. Proteases		
a. Rennin	Casein	Paracasein
b. Pepsin	Native proteins	Proteoses and peptones
c. Trypsin	Native proteins	Polypeptides and amino acids
d. Erepsin	Polypeptides	Amino acids
e. Papain	Native proteins	Polypeptides and dipeptides
f. Bromelin	Native proteins	Polypeptides and dipeptides
g. Keratinase	Keratins	Amino acids
h. Protaminases	Protamines with terminal arginine group	Arginine + residue
i. Aminopolypeptidases	Polypeptides with free amino group	Amino acids
j. Carboxypolypeptidases	Polypeptides with free carboxyl group	Amino acids
k. N-prolylpeptidase	Polypeptides containing the group —CO—N<	Proline + residue
l. Dipeptidases	Dipeptides	Amino acids

CLASSIFICATION OF ENZYMES—*Continued*

	Substrates	End Products
2. Desamidases		
a. Urease	Urea	Carbon dioxide + ammonia
b. Asparaginase	Asparagine	Aspartic acid + ammonia
c. Arginase	Arginine	Urea + ornithine
d. Histozyme	Hippuric acid	Benzoic acid + glycine
e. Guanosin desamidase	Guanosine	Guanine + pentose
f. Adenosin desamidase	Adenosine	Adenine + pentose
g. Xanthosinase	Xanthosine	Xanthine + pentose
h. Inosinase	Inosine	Hypoxanthine + pentose
i. Guanase	Guanine	Xanthine + ammonia
j. Adenase	Adenine	Hypoxanthine + ammonia
B. Desmolyzing enzymes		
I. Zymase	Hexose	Ethanol + carbon dioxide
II. Glycolase	Hexose	Lactic acid
III. Decarboxylase	R·CO·COOH	R·CHO + CO_2
IV. Enzymes involved in oxidation-reduction		
1. Alcohol dehydrogenase	Ethanol	Acetaldehyde
2. Acid dehydrogenases		
a. Succinic dehydrogenase	Succinic acid	Fumaric acid
b. Malic dehydrogenase	Malic acid	Pyruvic acid
c. Lactic dehydrogenase	Lactic acid	Pyruvic acid
d. β-Hydroxybutyric dehydrogenase	β-Hydroxybutyric acid	Acetoacetic acid
e. Citric dehydrogenase	Citric acid	Acetone dicarboxylic acid
f. Glycerolphosphoric dehydrogenase	Glycerolphosphoric acid	Glyceric aldehyde phosphoric acid
g. Glyceric aldehyde phosphoric acid dehydrogenase	Glyceric aldehyde phosphoric acid	Glyceric acid phosphoric acid
3. Glucose dehydrogenase	Glucose	Gluconic acid
4. Purinoxidases	(a) Hypoxanthine	Xanthine
	(b) Xanthine	Uric acid
	(c) Uric acid	Allantoin + carbon dioxide
5. Oxidoreductase	R·CHO	$R·CH_2OH$ + R·COOH
6. Glyoxalase	Methylglyoxal	Lactic acid
7. Reductases	(a) Methylene blue	Leuco base
	(b) Nitrates	Nitrites
8. Phenolases	Phenols	Quinones
a. Laccase	Lac	Lacquer
b. Tyrosinase	Tyrosine	Black pigments
c. Tyraminase	Tyramine	p-Oxyphenyl acetic acid + ammonia
d. Dopaoxidase	Dopa (dihydroxyphenylalanine)	Black pigments

CLASSIFICATION OF ENZYMES—*Continued*

	Substrates	End Products
9. Glucose oxidase		
a. Yellow oxidase (Warburg and Christian)	Hexose monophosphate (Robison)	Carbon dioxide + water
10. Luciferase	Luciferin	Oxyluciferin and light produced by bioluminescent organisms
V. Catalase	H_2O_2	$H_2O + O_2$
VI. Carbonic anhydrase	H_2CO_3	$CO_2 + H_2O$

THE CHEMICAL NATURE OF ENZYMES.—There are two schools of thought with regard to the chemical nature of enzymes. Willstätter,[19] as the result of many years of research in the purification of enzymes, enunciated in 1922 his "Träger" theory which has been accepted by Oppenheimer,[20] Waldschmidt-Leitz,[21] and most of the European workers. The viewpoint of this group is that enzymes contain a special, reactive group which either combines with, or possesses some particular affinity for, definite groupings in the substrate, thus accounting for the specificity of enzyme behavior. This special reactive group is attached to a colloidal carrier, and enzyme action is determined in part by the affinity of the active group for the substrate and in part by the colloidality of the entire aggregate. When the colloidal properties of the aggregate are destroyed, then the activity of the enzyme disappears. Willstätter in his earlier statements indicated that a single colloidal carrier did not appear to be essential but that any "suitable" colloidal carrier could act as a protective colloid for the active prosthetic group. This viewpoint has been more or less modified so that certain of the proponents of the "Träger" theory now suggest that the specificity of enzyme action is determined not alone by the nature of the prosthetic group but likewise by a specific colloidal carrier which shows specific affinities for the substrate.

Fodor[22] emphasizes even more strongly the importance of colloidality as determining enzyme action. He believes that enzymes are nothing more or less than commonly known substances, proteins, carbohydrates, etc., in a peculiar colloidal state, the activities being

[19] Willstätter, R., Über Isolierung von Enzymen, *Ber.*, 55: 3601–3623 (1922); *cf.* also Willstätter, R., Graser, J., and Kuhn, R., Zur Kenntnis des Invertins, *Z. physiol. Chem.*, 123: 1–78 (1922) (Note particularly pp. 45–60); Willstätter, R., Über Fortschritte in der Enzymisolierung, *Ber.*, 59: 1–12 (1926); and Zur Frage der proteinartigen Natur der Saccharase, *Ber.*, 59: 1591–1594 (1926).

[20] Oppenheimer, C., Die Fermente und ihre Wirkungen, Vol. I, Fifth Edition, Georg Thieme, Leipzig (1925).

[21] Waldschmidt-Leitz, E., Enzyme Actions and Properties, Translated and Extended by R. P. Walton, John Wiley & Sons, New York (1929).

[22] Fodor, A., Das Fermentproblem, Theodor Steinkopff, Dresden and Leipzig (1922).

due solely to the energies characteristic of colloidal systems. It is difficult to see, however, how enzyme specificity can be accounted for on the basis of Fodor's view.

The opposing viewpoint is typified by the American workers. The viewpoint here is that enzymes are specific and definite chemical compounds, probably proteins, and that the arrangement of the groupings in the protein molecule not only is responsible for the enzyme activity but likewise determines enzyme specificity.

Willstätter in his attempts to purify enzymes, and particularly with respect to the nature of invertase, makes the very definite statement that the enzyme does not belong to any of the usual groups, such as proteins, carbohydrates, lipids, etc., and that, so far as the chemical nature of the enzyme is concerned, our knowledge shrinks to the generalization that they are demonstrable only by their activity. Several workers, however, have pointed out that enzymatic activity can be demonstrated in solutions which are so exceedingly dilute that the usual tests for proteins, carbohydrates, etc., are lacking but that, in more concentrated solutions, positive protein tests can be obtained.

Sherman,[23] as a result of a long series of studies on pancreatic and malt amylases, states definitely that both these enzymes are proteins. Within recent years we have seen the successful preparation of a considerable number of "crystalline enzymes," and all of them have been shown to be proteins, or at least the colloidal carrier is a crystalline protein. The first crystalline enzyme was urease obtained by Sumner[24] in 1926. This enzyme, which Sumner describes as a globulin, was obtained from the jack bean and crystallizes in beautiful colorless octahedra. The enzymatic activity remains constant through repeated recrystallization, and the crystalline enzyme is so active that 1 gram will produce 100,000 mg. of ammonia from urea phosphate in 5 minutes at 20° C. or will decompose its own weight of urea in 1.4 seconds.

Pepsin was crystallized by Northrop[25] in 1929. Trypsin was

[23] Sherman, H. C., The Chemical Nature of Two Typical Enzymes.—Pancreatic and Malt Amylases, *Proc. Natl. Acad. Sci.*, U. S., 9: 81–86 (1923).

[24] Sumner, J. B., The Isolation and Crystallization of the Enzyme Urease, Preliminary Paper, *J. Biol. Chem.*, 69: 435–441 (1926); The Recrystallization of Urease, *ibid.*, 70: 97–98 (1926); Sumner, J. B., and Hand, D. B., Crystalline Urease, II, *ibid.*, 76: 149–162 (1928); Sumner, J. B., and Holloway, R. G., Crystalline Urease, III, *ibid.*, 79: 489–492 (1928); and Sumner, J. B., and Hand, D. B., The Isoelectric Point of Crystalline Urease, *J. Am. Chem. Soc.*, 51: 1255–1260 (1929).

[25] Northrop, J. H., Crystalline Pepsin, *Science*, 69: 580 (1929); Crystalline Pepsin, I. Isolation and Tests for Purity, *J. Gen. Physiol.*, 13: 739–766 (1930); II. General Properties and Experimental Methods, *ibid.*, 13: 767–780 (1930); III. Preparation of Active Crystalline Pepsin from Inactive Denatured Pepsin, *ibid.*, 14: 713–724 (1931); IV. Hydrolysis and Inactivation by Acid, *ibid.*, 16: 33–40 (1932); V. Isolation of Crystalline Pepsin from Bovine Gastric Juice, *ibid.*, 16:

crystallized by Northrop [26] in 1931. Chymotrypsin and chymotrypsinogen were crystallized by Kunitz and Northrop [27] in 1933. Trypsinogen was crystallized by Kunitz and Northrop [28] in 1934. A crystalline lipase was reported by Bamann and Laeverenz [29] in 1934. The yellow oxidation enzyme of Warburg and Christian was crystallized by Theorell [30] in 1935. Carboxypolypeptidase from the bovine pancreas, an enzyme which hydrolyzes polypeptides containing a free carboxyl group, was crystallized by Anson [31] in 1935. He describes this enzyme as a globulin. It is interesting to note that it attacks polypeptides and hydrolyzes them even in the presence of formaldehyde. In 1936, Herriott and Northrop [32] announced the isolation of crystalline pepsinogen from the gastric mucosa of swine, and, in 1937, Balls, Lineweaver and Thompson [33] obtained crystalline papain.

615–623 (1933); and Herriott, R. M., and Northrop, J. H., Crystalline Acetyl Derivatives of Pepsin, *ibid.*, 18: 35–67 (1934). See also Steinhardt, J., The Stability of Crystalline Pepsin, *Kgl. Danske Videnskab. Selskab, Math.-fys. Medd.*, 14: (No. 11) 53 pp. (1937).

[26] Northrop, J. H., and Kunitz, M., Isolation of Protein Crystals Possessing Tryptic Activity, *Science*, 73: 262-263 (1931); Crystalline Trypsin, I. Isolation and Tests of Purity, *J. Gen. Physiol.*, 16: 267–294 (1932); II. General Properties, *ibid.*, 16: 295–311 (1932); Experimental Procedure and Methods of Measuring Activity, *ibid.*, 16: 313–321 (1932); Northrop, J. H., IV. Reversibility of the Inactivation and Denaturation of Trypsin by Heat, *ibid.*, 16: 323–337 (1932); V. Kinetics of the Digestion of Proteins with Crude and Crystalline Trypsin, *ibid.*, 16: 339–348 (1932); and Kunitz, M., and Northrop, J. H., Inactivation of Crystalline Trypsin, *ibid.*, 17: 591–615 (1934).

[27] Kunitz, M., and Northrop, J. H., Isolation of a Crystalline Protein from Pancreas and its Conversion into a New Crystalline Proteolytic Enzyme by Trypsin, *Science*, 78: 558–559 (1933); and Crystalline Chymotrypsin and Chymotrypsinogen, I. Isolation, Crystallization, and General Properties of a New Proteolytic Enzyme and its Precursor, *J. Gen. Physiol.*, 18: 433–458 (1935).

[28] Kunitz, M., and Northrop, J. H., The Isolation of Crystalline Trypsinogen and its Conversion into Crystalline Trypsin, *Science*, 80: 505–506 (1934); and Isolation from Beef Pancreas of Crystalline Trypsinogen, Trypsin, a Trypsin Inhibitor, and an Inhibitor-Trypsin Compound, *J. Gen. Physiol.*, 19: 991–1007 (1936).

[29] Bamann, E., and Laeverenz, P., Über pankreatische Lyo- und Desmolipasen IV. Zur Kenntnis zellgebundener Enzyme der Gewebe und Drüsen in der von R. Willstätter und M. Rohdewald begonnenen Untersuchungsreihe, *Z. physiol. Chem.*, 223: 1–20 (1934).

[30] Theorell, H., Das Gelbe Oxydationsferment, *Biochem. Z.*, 278: 263–290 (1935).

[31] Anson, M. L., Crystalline Carboxypolypeptidase, *Science*, 81: 467–468 (1935); and Carboxypeptidase, I. The Preparation of Crystalline Carboxypeptidase, *J. Gen. Physiol.*, 20: 663–669 (1937).

[32] Herriott, R. M., and Northrop, J. H., Isolation of Crystalline Pepsinogen from Swine Gastric Mucosae and its Autocatalytic Conversion into Pepsin, *Science*, 83: 469–470 (1936).

[33] Balls, A. K., Lineweaver, H., and Thompson, R. R., Crystalline Papain, *Science*, 86: 379 (1937).

Table LXXXIII lists some of the characteristic properties of these crystalline enzymes as reported by the various authors. The data reported for urease, pepsinogen, papain, and lipase are inadequate for

TABLE LXXXIII

SOME OF THE PROPERTIES OF THE CRYSTALLINE ENZYMES*

	Pepsin	Trypsin	Trypsinogen	Chymotrypsin	Chymotrypsinogen	Carboxypeptidase	Yellow Oxidation Enzyme
Crystalline Form.....	Dihexagonal bipyramids	Colorless, short, rectangular prisms	Short, triangular prisms	Colorless rhombohedrons	Colorless, long, square prisms	Colorless, long prisms	Short, cubical prisms
Elementary Analysis... C.....	52.4	50.0	50.1	50.0	50.6	52.6	51.5
H.....	6.67	7.1	6.9	7.06	7.0	7.2	7.37
N.....	15.3	15.0	15.3	15.5	15.8	14.4	15.9
Cl.....	0.22	2.85	.....	0.16	0.17	.....	.....
S.....	0.86	1.1	1.1	1.85	1.9	0.47	.....
P.....	0.078	0.0	0.0	0.0	0.0	0.00	0.043
Fe.....	.....	.....	.....	.....	.....	.....	.....
Ash.....	0.40	1.0	.....	0.12	0.1	0.68	.....
Amino N as % total N	5.26	9.3	.....	6.00	4.75	.....	.....
Diffusion coefficient cm.²/day.....	0.047	0.023	.....	0.037	0.039	.....	.....
Particle weight.....	37,000	36,500	.....	41,000	36,000	.....	70–75,000
Isoelectric point (*p*H).	2.75	7–8	.....	5.4	5.0	.....	5.2
Optimum activity † (*p*H).....	2.75–3.0	8–9	None	8–9	None	.....	5–6

* Urease, pepsinogen, papain, and lipase have meager analytical data. Urease crystallizes in colorless octahedra, contains approximately 17 per cent of nitrogen, has an isoelectric point at *p*H 5.0–5.1, and optimum activity at *p*H 7.0. Pepsinogen crystallizes in colorless needles and has no proteolytic activity. Lipase and papain crystallize in colorless needles.

† Under experimental conditions of the investigation.

a complete chemical characterization. Northrop [34] in particular insists upon the viewpoint that his crystalline enzymes are specific proteins.

The Träger theory was adopted by Willstätter before there was any definite evidence as to the type of compound which might constitute the prosthetic group. More recently it has been shown [35] that the prosthetic group in cytochrome-c is an iron-porphyrin residue, probably identical with the iron-porphyrin of hemoglobin. Furthermore the prosthetic group in liver catalase is an iron-porphyrin compound [36] and an iron-porphyrin residue constitutes the prosthetic group

[34] Northrop, J. H., The Formation of Enzymes, *Physiol. Rev.*, 17: 144–152 (1937).

[35] Hill, R., and Keilin, D., The Porphyrin of Component C of Cytochrome and its Relationship to Other Porphyrins, *Proc. Roy. Soc.* (*London*), B 107: 286–292 (1930); and Zeile, K., and Reuter, F., Über Cytochrom c, *Z. physiol. Chem.*, 221: 101–116 (1933).

[36] Zeile, K., and Hellström, H., Über die aktive Gruppe der Leberkatalase, *Z. physiol. Chem.*, 192: 171–192 (1930); Zeile, K., Über die aktive Gruppe der Katalase, II, *ibid.*, 195: 39–48 (1931); and Stern, K. G., The Constitution of the Prosthetic Group of Catalase, *J. Biol. Chem.*, 112: 661–669 (1936).

in peroxidase.[37] The evidence points to the same iron-porphyrin residue being present in these three enzymes, but in each instance the porphyrin residue is associated with a specific protein and therefore the characteristics of the enzyme and its specificity are determined not alone by the prosthetic group but likewise by the specific protein with which the prosthetic group is associated. In the case of catalase the iron-porphyrin residue, hematin, will act by itself to decompose hydrogen peroxide, but the action is exceedingly feeble. When the iron-porphyrin residue is associated with the specific protein which accompanies it in the enzyme catalase, the combination is approximately ten million times more active in decomposing hydrogen peroxide than is the porphyrin residue alone. Therefore in these three proteins at least there are a non-protein residue and a colloidal carrier in the sense of the Träger theory, both being essential parts of the enzyme molecule.

Perhaps an even more striking example is the yellow oxidation enzyme of Warburg and Christian. We have already discussed this system in the chapter on flavins. The prosthetic group is a riboflavinphosphoric acid ester. This group has no enzymatic activity of itself. In the yellow oxidation enzyme the riboflavinphosphoric acid ester is associated with a specific protein. The specific protein has no enzymatic activity of itself. The two components, when combined in the proper proportions, regenerate the original enzymatic activity, as shown by Fig. 159, where varying quantities of riboflavinphosphoric acid ester were added to a constant quantity of the specific protein originally separated from the yellow oxidation enzyme. It will be noted that the activity of the mixture increases in direct proportion to the amount of prosthetic group which is added until a definite activity is reached past which point the addition of further riboflavinphosphoric acid ester caused no further increase in activity. The recombination of the specific protein and the riboflavinphosphoric acid ester by Theorell is a definite synthesis of an enzyme and bears out the Träger theory of Willstätter.

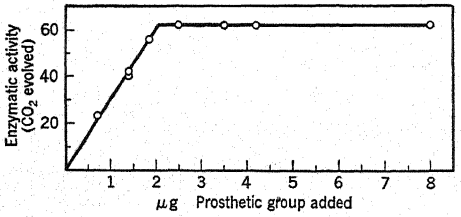


FIG. 159.—Showing the effect of adding varying amounts of the prosthetic group to the specific protein in resynthesizing the yellow oxidation enzyme. (After Theorell.)

The viewpoints of the two groups are excellently summed up in

[37] Kuhn, R., Hand, D. B., and Florkin, M., Über die Natur der Peroxydase, *Z. physiol. Chem.*, 201: 255–266 (1931).

general papers by Waldschmidt-Leitz [38] and Sumner.[39] Waldschmidt-Leitz cites the catalase and peroxidase systems noted above and insists that the crystalline enzymes which have been designated as proteins are adsorption compounds of a true enzymatic component plus a crystalline protein for which the prosthetic group has particular affinities. Sumner very pertinently asks whether the enzymatic behavior should be attributed to a part of the enzyme or whether the whole complex should be taken into consideration and likewise whether one part of an enzyme can be more important than another. If the catalytic activity of hematin increases ten million fold when associated with the colloidal carrier, is not the colloidal carrier of at least equal importance with the prosthetic group?

In pepsin, trypsin, carboxypolypeptidase, urease, etc., no specific prosthetic group has been discovered. Northrop appears to believe that the enzymatic activity is due to a particular arrangement of the amino acids in the molecule in much the same way that the activity of insulin appears to be due to specific amino-acid groupings. If this be so, it will probably be very difficult to elucidate the nature of the specific groupings responsible for enzymatic behavior in these systems. On the other hand, the possibility of a true prosthetic group in the sense of Willstätter has not been definitely ruled out. In catalase, peroxidase, and the yellow oxidation enzyme, the prosthetic group can be separated from the specific protein by physical technics. The linkages between the specific group and the protein are either of the nature of salt formation or adsorption affinities. It may well be that in some of the other proteins actual carbon-to-carbon or carbon-to-nitrogen bonds link the prosthetic group to the protein molecule. In that case destruction of the protein would accompany the breaking of such bonds, and it would be very difficult to isolate the protein and the prosthetic group, to show that each by itself was inactive, and then to recombine them as Theorell did with the complete regain of enzymatic activity.

At the present time sufficient definite evidence is available to demonstrate that the Träger theory of Willstätter, with minor modifications, does account for the enzymatic behavior of certain enzymes. Possibly other enzymes are specific proteins. The preparation of enzymes in the crystalline form with constant activities unchanged by repeated recrystallization represents an enormous advance in the elucidation of problems concerning enzyme action, and we can only hope that an intensive study of these compounds will some day afford a clew to the nature of enzyme action.

[38] Waldschmidt-Leitz, E., The Chemical Nature of Enzymes, *Science*, 78: 189–190 (1933).

[39] Sumner, J. B., The Chemical Nature of Enzymes, *Science*, 78: 335 (1933).

INTERMEDIATE PRODUCTS BETWEEN ENZYME AND SUBSTRATE.—Most theories of enzyme action have postulated a union of enzyme and substrate to form an intermediate product which breaks down with the regeneration of the enzyme and substrate decomposition products.

(1) Enzyme + substrate $\rightleftharpoons$ enzyme-substrate compound
(2) Enzyme-substrate compound $\rightarrow$ enzyme + product molecules

Stern [40] has given definite proof of an intermediate compound between catalase and monoethyl hydrogen peroxide. The system was studied spectrophotometrically.

(1) The purified catalase enzyme yields a brown solution with characteristic absorption bands at 650, 646-620, and 610 mμ.

(2) When ethyl hydrogen peroxide is added to the catalase solution, there is a short period when the brown color characteristic of the enzyme disappears and a pale green color develops. During this stage the absorption bands of catalase disappear and no new characteristic absorption bands develop.

(3) Following the initial period there develops a red color of the catalase-substrate compound. This intermediate compound has very pronounced absorption bands at 576-564 and 540-529 mμ.

(4) As the red catalase-substrate compound breaks down, there coexists in the solution the absorption spectrum of both the free enzyme and the enzyme-substrate compound.

(5) When all the enzyme-substrate compound has been broken down, the brownish color characteristic of the enzyme and the characteristic enzyme absorption bands alone remain.

The above cycle is repeated when fresh substrate is added to the enzyme solution.

It seems probable that similar enzyme-substrate combinations take place as intermediary compounds in most enzyme reactions. The difficulty has been to demonstrate the presence of specific compounds inasmuch as they may exist for only short periods of time.

THE KINETICS OF ENZYME ACTION.—The law of mass action involving the transformation of one compound into another states that the rate of reaction must be proportional to the concentration of the reacting molecules. Thus, if a molecule of maltose is undergoing the process of hydrolysis, giving rise to two molecules of glucose, the rate of hydrolysis must be proportional to the concentration of the maltose present and to the amount of glucose already formed. Inasmuch as

[40] Stern, K. G., On the Mechanism of Enzyme Action. A Study of the Decomposition of Monoethyl Hydrogen Peroxide by Catalase and of an Intermediate Enzyme-Substrate Compound, *J. Biol. Chem.*, 114: 473–494 (1936).

such reactions ordinarily take place in the presence of a great excess of water, the change in the concentration of the water is usually ignored, and while one molecule of water is taken up for each molecule of sugar which is hydrolyzed, the small amount of water which is used does not appreciably alter the water concentration. The transformation of a molecule of maltose by hydrolysis into two molecules of glucose is what is known as a monomolecular reaction. Such a reaction may be expressed by the equation

$$v = \frac{dx}{dt} = K(a - x) \tag{186}$$

where v = the rate of the reaction at time t;
x = the amount of substance which has already been transformed in time t;
$(a—x)$ = the concentration of the material which is being transformed, a being the initial concentration of the substance;
K = a constant.

This equation states that *the rate of reaction is proportional to the concentration at any specific time.* It has been abundantly demonstrated that an equation of this type fits the usual hydrolytic reactions, such as are involved in the catalytic inversion of cane sugar in the presence of hydrogen ions, the catalytic inversion of maltose, lactose, etc. By integrating equation (186) we obtain the equation

$$K = \frac{1}{t} \log_e \frac{a}{(a - x)} \tag{187}$$

which can again be expressed as

$$K = \frac{1}{(t_2 - t_1)} \log_e \frac{C_1}{C_2} \tag{188}$$

where C_1 and C_2 = the concentrations of the substrate at time t_1 and t_2, respectively, the time being taken from the beginning of the reaction.

In the last two equations we are dealing with a logarithmic curve where one set of coordinates is a series of numbers and the other set is the logarithms of numbers. Equation (188) makes it possible to determine the velocity constant in the original equation (186) from any two determinations of the concentration of the substrate at any two intervals of time.

The validity of these monomolecular reaction equations has been demonstrated for certain enzymatic reactions. The author believes that most of the discrepancies between the experimental and theoretical (calculated) data based upon a monomolecular formula are

due not to the actual divergence of the reaction from a monomolecular reaction but rather to uncontrolled, and in many instances uncontrollable, changes which occur in the system under investigation. That is to say, if all variables excepting the concentration of the substrate could be held constant over the range t_0 to t_1 to t_2, it is entirely probable that the monomolecular laws would be found to hold rigidly. However, it is impossible to demonstrate that there has been no alteration in the activity of the enzyme preparation during the course of the experiment. If the activity of the enzymes is increased, there will be a deviation from the expected results. If any proportion of the enzyme is inactivated or destroyed, this again will cause a deviation from the theoretical values. In studies where we deal only with the concentration of hydrogen ions, it is possible to set up a univariant system. No biological system as yet realized is absolutely univariant, and until such systems can be set up and accurately controlled, we must expect at the best to reach only approximations of the theoretical results.

We have already noted the theory that enzymatic action is preceded by a combination of enzyme and substrate to form an unstable compound which breaks down, regenerating the enzyme and yielding split products. Brown [41] suggested that invertase action was proportional to the concentration of the enzyme-substrate compound and that invertase combines with sucrose, molecule for molecule, according to simple mass law. This was tested experimentally by Michaelis and Menten,[42] who found that the reaction could be expressed by the equation:

$$\frac{I_2}{I_1} = \frac{S}{S + K_s} \tag{189}$$

where I_1 = total invertase concentration;
I_2 = combined invertase concentration;
S = concentration of free sucrose;
K_s = dissociation constant of intermediate compound.

Michaelis and Menten showed that the experimental results agreed very closely with the theoretical mass-law curve when the concentrations of sucrose were below 5 per cent but that there was a great deviation of the experimental from the theoretical values at higher sucrose concentrations. They postulated that the deviation at higher sucrose concentrations was due to the fact that all the enzyme had been combined with sucrose below the 5 per cent sucrose concentration and accordingly the sucrose added in excess of this amount could not

[41] Brown, A. J., Enzyme Action, *J. Chem. Soc.*, 81: 373–388 (1902).
[42] Michaelis, L., and Menten, Maud L., Die Kinetik der Invertinwirkung, *Biochem. Z.*, 49: 333–369 (1913).

influence the rate of reaction. Later, however, Nelson and Schubert [43] showed that the falling off of enzymatic activity at the higher sucrose concentrations was due predominantly to a decrease in the water concentration in the system, which factor had not been taken into account by the earlier workers.

Various equations have been devised to express the course of enzyme action under definite experimental conditions. Some of these equations are purely empirical; others are based upon the mass action laws. Schütz [44] announced that the rate of hydrolysis of egg albumin by pepsin in a given time was proportional to the square root of the concentration of the enzyme. Northrop,[45] however, has shown that only free pepsin is involved in the reaction and that the hydrolytic products tend to "combine" with pepsin and thus prevent further hydrolysis by that fraction of the pepsin with which they are combined. Northrop's equation for peptic hydrolysis is

$$K = \frac{A \log_e \left(\frac{A}{A - x}\right) - x}{Et} \tag{190}$$

where K = the reaction constant;
A = original total concentration of substrate;
x = amount of substrate hydrolyzed in time t;
E = concentration of enzyme.

Thus, as the reaction progresses, the free pepsin available for further reaction decreases.

One additional factor comes into play in dealing with enzymatic reactions. We have already noted that in many instances hydrolysis and synthesis may be expressed by a reversible reaction [46] and have already set up the hydrolytic equilibrium

$$\text{Glucoside} + \text{water} \rightleftharpoons \text{sugar} + \text{non-sugar}$$

The reaction reading from left to right is a monomolecular reaction. The reaction reading from right to left is a bimolecular reaction. The velocity constant for the synthetic reaction will not be identical with

[43] Nelson, J. M., and Schubert, M. P., Water Concentration and the Rate of Hydrolysis of Sucrose by Invertase, *J. Am. Chem. Soc.*, 50: 2188–2193 (1928); *cf.* also Nelson, J. M., Enzymes from the Standpoint of the Chemistry of Invertase, *Chem. Rev.*, 12: 1–42 (1933).

[44] Schütz, E., Eine Methode zur Bestimmung der relativen Pepsinmenge, *Z. physiol. Chem.*, 9: 577–590 (1885).

[45] Northrop, J. H., The Effect of the Concentration of Enzyme on the Rate of Digestion of Proteins by Pepsin, *J. Gen. Physiol.*, 2: 471–498 (1920).

[46] *Cf.* also Borsook, H., Reversible and Reversed Enzymatic Reactions, *Ergebn. Enzymforsch.*, 4: 1–41 (1935).

that for the hydrolytic reaction. The synthetic reaction may be expressed as

$$\frac{dx}{dt} = K_2x^2 \tag{191}$$

where x = the concentration of either the uncombined sugar or the uncombined non-sugar radical at time t.

Inasmuch as these were originally present in equivalent quantities and they combine in equal proportions, the concentration of each can be designated by the same symbol. Accordingly, if any tendency toward a synthetic reaction occurs when enzyme phenomena are under investigation, it would be necessary to provide for such a possibility by including in the equation both the hydrolytic and the synthetic velocity constants. Such a possibility has been discussed by Moore,[47] who suggests the equation

$$\frac{dx}{dt} = K_1(a - x) - K_2x^2 \tag{192}$$

where K_1 = the velocity constant of the hydrolytic process, and
K_2 = the velocity constant of the synthetic process.

Moore goes further than this and adds to the equation an expression for any change which may have taken place in the activity of the enzyme preparation during the time of the experiment. However, inasmuch as it is extremely difficult to demonstrate the rate of alteration of enzymatic activity, we will not include his expressions at this point, the reader being referred to his papers for that discussion.

The above discussion has been based upon the assumption that enzymatic reactions obey the physicochemical laws of true solutions. As we have already noted in the discussion of the colloid state, our knowledge of the energies of colloid systems is in most instances too inadequate to draw final conclusions and to fit colloidal behavior completely into the scheme of the classical physicochemical systems. Accordingly it is not surprising that most workers have found enzymatic reactions to deviate somewhat from the theoretical as calculated by the above equations.

Rate of diffusion undoubtedly plays a very important role in any system of chemical kinetics. When we are dealing with colloidal systems or when colloidal phases are present, the rate of diffusion of the colloid may be infinitely low, so that only one of the components of the reaction diffuses at an appreciable rate. Thus, if Willstätter's views

[47] Moore, B., Velocity of Reaction, and the Comparative Action of Enzymes and Cells—Experimental Observations on Velocity of Reaction, and Their Discussion—Alteration of Concentration of Enzyme, Chapter III of Recent Advances in Physiology and Bio-Chemistry, edited by Leonard Hill, Edward Arnold, London (1908).

are correct, *i.e.*, that enzymes possess a chemically reactive group, we must expect the reactions taking place in such a system to be somewhat slowed down, inasmuch as the molecule which is being hydrolyzed must diffuse to the reactive group, and after hydrolysis has taken place, the hydrolytic products must diffuse out from the reactive group in order to allow additional substrate to come in contact at the interface. This factor alone may be sufficient to account for some of the divergences which have been observed in enzyme studies.

Adding to this the possibility that the hydrolytic products, or the substrate itself, or even the water which makes up the dispersions medium may alter continuously or discontinuously the colloidality of the enzyme itself, only illustrates the complexity of the problem of enzyme kinetics. We have emphasized again and again the ease with which colloidal systems may undergo profound alteration. It is not surprising, therefore, that the environment of the enzyme profoundly affects not only the rate of reaction but the maintenance or the rapid destruction of enzyme activity. Accordingly the presence or absence of traces of various substances may profoundly change the course of an enzyme reaction. A few instances of this sort will be discussed in the succeeding sections. Presumably it is the maintenance or the destruction of the colloidality of the carrier of the reactive group which is influenced, or the irreversible adsorption of some compounds upon the "active centers" of the enzyme, which compounds are not "activated" and therefore block other molecules, which can be activated, from reaching the active centers. We have noted this phenomenon in discussing Quastel's studies of adsorption and activation on the bacterial mosaic.

In the study of rates of reactions in homogeneous systems, great emphasis has been laid upon the fact that reliable results cannot be obtained except when all factors are held constant excepting the one variable which is being studied. Unfortunately we have not yet reached the point in enzyme chemistry where we can realize this ideal. However, it should be the goal toward which we should strive, and so far as possible enzyme studies should be conducted with the viewpoint of studying systems possessing only a single variable. The importance of certain of the more significant variables is noted in the succeeding sections.

Hydrogen-Ion Concentration.—Inasmuch as enzymes appear to have the properties of lyophilic colloids, it might be expected that their activity would be profoundly influenced by hydrogen-ion concentration. This expectation agrees with experimental findings. Enzyme preparations, in general, show a definite optimum hydrogen-ion concentration at which the rate of reaction may be many times greater than at other different hydrogen-ion concentrations. Thus, the proteolytic enzyme, pepsin, shows its optimum activity in a highly acid

solution approximating a *p*H of 2.5. Trypsin, on the other hand, has its greatest activity in a nearly neutral or faintly alkaline solution, approximately *p*H 8.0. Figure 160 [48] shows the relationship between the activity of the amylases of wheat flour and the hydrogen-ion concentration of the medium. In these experiments 10-gram samples of wheat flour were added to 100 cc. of distilled water which had already been brought to temperature, and the samples were allowed to digest at 27° C. for 1 hour. It will be noted that there is an increase of more than 500 per cent in the rate of reaction at a *p*H of 5.00, as contrasted to the activity at *p*H 7.00. Similar curves have been plotted by various workers for many of the enzymes. In general, the

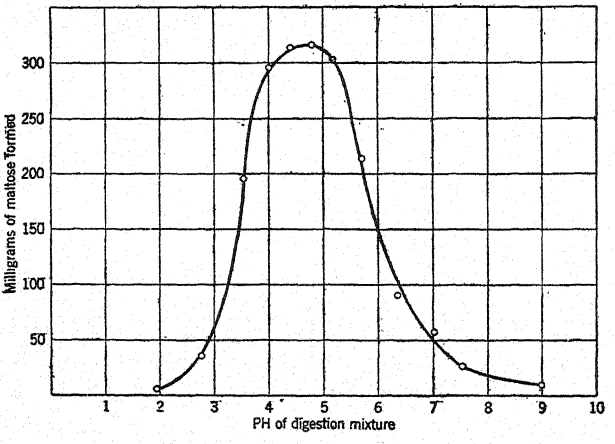


FIG. 160.—Showing the influence of hydrogen-ion concentration on the activity of wheat-flour amylase. Ten grams of wheat flour autodigested at 27° for 1 hour. (Data of Rumsey.)

form of the curve is very similar to that shown in Fig. 160, although the point at which the maximum activity is reached may be shifted, depending upon the enzyme which is being studied.

Particular attention should be called to the extremely steep slope of the curve away from the optimum area on both the ascending and descending sides. It is evident that a very slight change in hydrogen-ion concentration on this portion of the curve might readily account for very great changes in activity.

We have noted the probability that at least in certain instances enzyme reactions represent reversible systems and that both synthetic

[48] Rumsey, L. A., The Diastatic Enzymes of Wheat Flour and Their Relation to Flour Strength, Ph.D. thesis, University of Minnesota, published as Bulletin 8, The American Institute of Baking (1922).

and hydrolytic reactions may occur simultaneously. Wilcox[49] interprets his study of urease activity over a range of hydrogen-ion concentration as showing a predominating tendency for the converting of urea into ammonium carbonate toward the alkaline side of the isoelectric range of the enzyme, whereas on the acid side of the isoelectric range the tendency of the enzyme was to synthesize urea from ammonium carbonate. Thus a shift in the hydrogen-ion concentration may very greatly alter the position of equilibrium between the synthetic and hydrolytic reactions.

NEUTRAL ELECTROLYTE CONCENTRATION.—In a number of enzyme systems the presence or absence of neutral electrolytes has a great influence on the activity of the enzyme. Pancreatic amylase can be taken as an example. Sherman[50] has shown that pancreatic amylase is practically inactive in the absence of neutral electrolytes and that it is very greatly activated by concentrations of sodium chloride as low as 0.0005 *M*. However, all neutral electrolytes do not activate pancreatic amylase to the same degree. Sodium sulfate and sodium phosphate were found to be essentially without effect, and a very definite lyotropic series of anions was demonstrated where the influence on amylase activity was $Cl > Br > NO_3 > ClO_3 > CNS > F > SO_4 = PO_4$. It was likewise found that the optimum concentration of neutral electrolyte might shift the optimum hydrogen-ion concentration to a different value. Certain of Sherman's data are shown in Table LXXXIV.

TIME.—In any biological system, time must be considered as of equal importance to any of the other variables. This fact has been either overlooked or ignored by many workers in the field of enzyme chemistry. Thus, for example, we read that some particular enzyme has a given "optimum" hydrogen-ion concentration, or that a given enzyme has an "optimum" temperature, or that it has an "optimum" for some other variable. The author wishes definitely to challenge every statement which has been made in regard to any "optimum condition" in which the variable, *time,* is not stated. *There can be no optimum hydrogen-ion concentration and no optimum temperature independent of time.* This is evident when we consider that every enzyme reaction taking place is a balanced reaction, the balance being between enzyme action and enzyme destruction. A given amount of enzyme is present at the beginning of the experiment. This amount of

[49] Wilcox, J. M., Relation Between the Isoelectric Range of Urease and the Reversibility of Its Action, *Proc. Soc. Exptl. Biol. and Med.,* 27: 228–231 (1929).

[50] Sherman, H. C., Caldwell, M. L., and Adams, M., The Interrelation of Hydrogen-Ion Activity and Concentration of Salt in the Activation of Pancreatic Amylase, *J. Am. Chem. Soc.,* 50: 2529–2535 (1928); The Influence of Concentration of Neutral Salt on the Activation of Pancreatic Amylase, *ibid.,* 50: 2535–2537 (1928); and A Quantitative Comparison of the Influence of Neutral Salts on the Activity of Pancreatic Amylase, *ibid.,* 50: 2538–2543 (1928).

TABLE LXXXIV

INCREASE IN ACTIVITY OF PURIFIED PANCREATIC AMYLASE AS INFLUENCED BY "OPTIMAL" CONCENTRATIONS OF VARIOUS NEUTRAL SALTS (ON BASIS OF NaCl EFFECT = 100)

(DATA OF SHERMAN, CALDWELL, AND ADAMS)

Salt Used	Optimal Concentration*	Optimal *p*H*	Relative Activity
	molar		
NaCl	0.02–0.05	7.1–7.2	100
KCl	0.03–0.05	7.1–7.2	100
LiCl	0.02–0.05	7.1–7.2	80–90
NaBr	0.03–0.20	7.1	77
$NaNO_3$	0.10–0.20	7.1	40
$NaClO_3$	0.10–0.20	6.9–7.1	27
NaCNS	0.15–0.20	6.7–6.8	28
NaF	0.20–0.30	6.7–6.8	21
Na_2SO_4	†	†	0
Na_3PO_4	†	†	0

* Under experimental conditions.
† No effect on activity over a wide range of concentrations and a wide *p*H range.

enzyme may well have changed during the course of the experiment. The rate of increase of enzyme activity and the rate of increase of enzyme destruction, so far as we know, bear no constant relationship. Accordingly, a change to one hydrogen-ion concentration may greatly speed up enzymatic activity and only slightly alter the rate of enzyme destruction. Changing to another hydrogen-ion concentration may maintain the same enzymatic activity or may cause a slight increase in activity at the same time greatly increasing the rate of enzyme destruction, so that the experimental results would indicate a decrease of enzyme action not due to a decreased enzyme activity but rather to an increased enzyme destruction. Inasmuch as we are always dealing with these two opposing forces, any optimum must be defined in terms of time.

Many of our textbooks are full of statements that the optimum temperature for enzymatic activity is approximately 37° C. This is probably true for pepsin or trypsin, and for most of the enzymes obtained from warm-blooded animal sources, providing that time is measured in hours. On the other hand, if time is measured in days, it may well be that the optimum temperature will approximate room temperature; or if time is measured in weeks, it may well be that the optimum temperature may approximate the icebox temperature; or if it is measured in minutes, it is not improbable that the optimum temperature may lie at 50°, 60°, or even 70° C.

Most enzymatic studies dealing with the hydrolysis of starch by amylases have been conducted at incubator temperature somewhere in the neighborhood of 37° C. In many instances, the time has been measured in hours. Figure 161, shows the combined effects of temperature and time upon the autodigestion of 10 grams of wheat flour suspended in 100 cc. of distilled water. This suspension had a *p*H of 5.7. Particular attention is directed to the curve showing the rate of maltose production at 55° C. The initial part of this curve is almost perpendicular, but after about 30 minutes the curve begins to flatten out; and at the end of 120 minutes has reached approximately 90 per cent of the height which is reached at the end of 360 minutes. In other words, the curve for the saccharogenic activity of the wheat-flour amylases is an expression of both temperature and time. The activity of the enzyme is enormously higher at 55°, but apparently enzyme destruction likewise proceeds very rapidly at this temperature, resulting in the inactivation of the enzyme which is present.

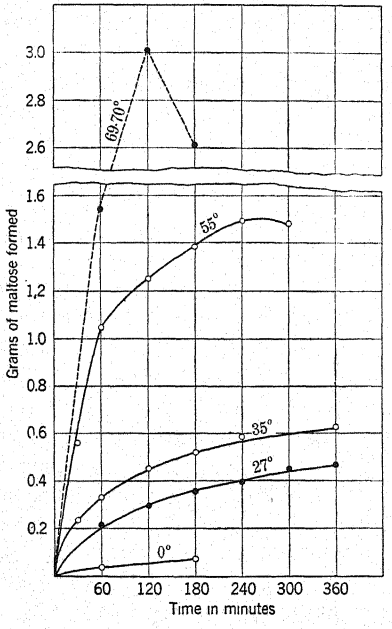


FIG. 161.—Showing the maltose formed by wheat-flour amylase acting upon raw wheat starch, with time and temperatures as variables. (Data of Rumsey.)

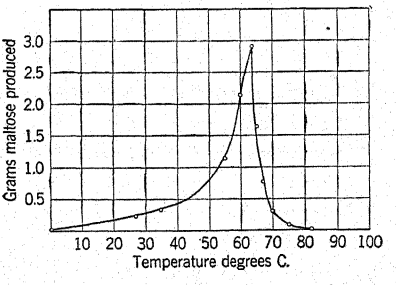


FIG. 162.—Showing the effect of temperature at constant time (1 hour) on maltose production from the autodigestion of 10 grams of wheat flour. (Data of Rumsey.)

This is perhaps more strikingly shown in Fig. 162, where time has been held constant and temperature is the only variable. Time in this figure is 1 hour, the other conditions of the experiment being as already noted. Certainly in this experiment the optimum temperature for the activity of the saccharogenic enzymes present in wheat flour approximates 60° C. However, immediately following this optimum temperature, there is a rapid fall in activity, indicating that enzyme

inactivation is progressing at an incomparably greater rate of speed than determines the rate of starch hydrolysis, *i.e.*, the enzyme is being inactivated before it can bring about any appreciable hydrolysis of the starch.

Figure 163 [51] shows a somewhat similar study dealing with the diastatic activity of wheat flours in the higher temperature ranges but with smaller increments of temperature change. This figure shows very definitely that optimum conditions are a reflection of both increase in enzyme activity and the rate of enzyme destruction, and that for wheat-flour amylases the optimum temperature for all times between 15 minutes and 1 hour is 63° C. However, at a time of 5 minutes or 10 minutes a higher optimum temperature is indicated.

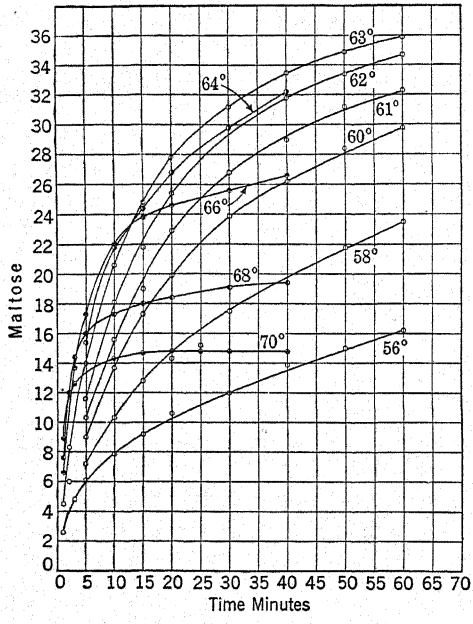


FIG. 163.—The diastatic activity of a wheat flour with time and temperature as variables. (Data of Munz and Bailey.)

Other and similar curves could be given, where hydrogen-ion concentration represents the only variable, and here again we would find different optimum hydrogen-ion concentrations for different times and for different temperatures. Sufficient illustrations have been given, however, to emphasize the fact that time must be regarded as one of the most important variables in enzyme studies.

TEMPERATURE.—We have noted in the preceding section a number of illustrations which in themselves are sufficient to emphasize the importance of temperature control in enzyme studies. Here again we must recognize that a change in temperature may well cause a shifting of the equilibria in respect to other environmental factors. This is particularly emphasized in the effect of temperature upon optimum

[51] Munz, Emil, and Bailey, C. H., Refractometric Methods of Determining Diastatic Activity of Flour, *Cereal Chem.*, 14: 85–96 (1937).

hydrogen-ion concentration. Figure 164[52] shows the effect of temperature on optimum hydrogen-ion concentration for the activity of malt amylase. It will be noted that there are apparently optimum hydrogen-ion concentrations of approximately *p*H 6.0 at 70°, approximately *p*H 5.0 at 45°, and approximately *p*H 4.3 at 25°. We have already noted in Fig. 162 the effect of temperature upon the activity of the saccharogenic enzymes present in wheat flour. Somewhat similar relationships between saccharogenic activity of malt flour and temperature are shown in Fig. 165.[53] In this curve, 10 grams of malt flour, suspended in 100 cc. of distilled water, were allowed to undergo autodigestion for a period of 1 hour. The curve shows several distinct portions. An almost linear relationship between temperature and diastase activity is shown in the area between 27° C. and 45° C.; the rate of activity greatly increases between 45° C. and 50° C. becomes almost linear between 50° C. and 60° C., still maintaining its increased activity, whereas at approximately 60° C. the activity begins to fall off very rapidly, and at temperatures above 65° C. the rate of enzyme destruction exceeds the rate of starch hydrolysis. Had a different period of time than 1 hour been chosen the characteristics of the curve would have been altered.

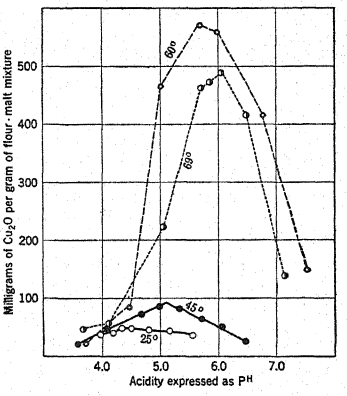


FIG. 164.—Showing the influence of temperature on the optimum hydrogen-ion concentration for the saccharogenic activity of malt. Time, 1 hr. (Data of Olsen and Fine.)

The fact that enzymes may possess extremely great activity at elevated temperatures is not generally emphasized. There are instances, however, where individual usage may be made of this fact. A certain sorghum syrup factory experienced difficulty in filtration of the defecated juice, due to the presence of traces of starch in the cane which became gelatinized when the juice was heated in the defecators. This gelatinized starch then passed with the juice to the filter presses, causing a clogging of the presses. The clogging of the presses could be prevented only by the use of relatively large quantities of infusorial

[52] Olsen, A. G., and Fine, M. S., Influence of Temperature on Optimum Hydrogen-ion Concentration for the Diastatic Activity of Malt, *Cereal Chem.*, 1: 215-221 (1924).

[53] Collatz, F. A., Flour Strength as Influenced by the Addition of Diastatic Ferments, Ph.D. thesis, University of Minnesota, published as Bulletin 9, The American Institute of Baking (1922).

earth. It was impracticable from the commercial standpoint to cool the juice after defecation to temperatures such as are ordinarily used for the hydrolysis of starch by means of enzymes. Accordingly an attempt was made to hydrolyze the starch in the defecated juice by the use of amyloclastic enzymes at temperatures exceeding 60° C. Additions of small amounts of such enzyme preparations solved the problem,[54] for although they were rapidly inactivated by the high temperature, the small amount of starch which was present was hydrolyzed, at least to the extent that no further trouble in filtration was experienced. Following the adoption of such a procedure, it was possible to dispense with more than half of the filter presses which had been previously employed and to reduce the amount of infusorial earth to a fraction of the amount which had previously been required.

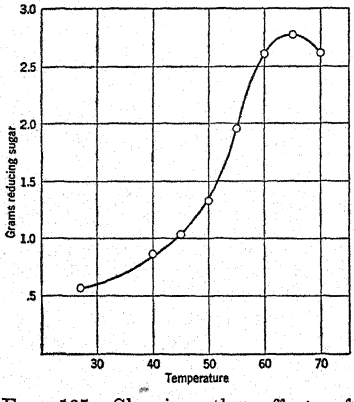


FIG. 165.—Showing the effect of temperature upon the activity of malt amylase when 10-gram portions of malt flour were autodigested for one hour. (Data of Collatz.)

The temperature coefficient for enzyme action follows more or less the van't Hoff rule that the activity is doubled for a 10° rise in temperature. Unfortunately, most of the studies which are available cover only a relatively small temperature range, and in many instances the values compiled from the work of different authors cannot be accurately interpreted because of the failure of the authors to record the hydrogen-ion concentration or other factors characteristic of the systems which they were studying. Values for Q_{10} as high as 5.30 have been reported for trypsin, and as low as 1.20 for certain of the lipases. These probably represent the extreme range for temperature coefficients, except near the temperature where inactivation is exceedingly rapid.

ACTIVATION AND INACTIVATION OF ENZYMES.—The literature of enzyme chemistry is replete with papers dealing with the influence of traces of added materials upon enzymatic activity. Perhaps the most consistent series of experiments are those which have been carried out by Sherman [55] and his co-workers. Sherman finds, in general, that

[54] Willaman, J. J., and Davison, F. R., Starch in Sorghum Sirup, *Ind. Eng. Chem.*, 16: 609–610 (1924).

[55] Sherman, H. C., and Walker, F., Influence of Aspartic Acid and Asparagin upon the Enzymic Hydrolysis of Starch, *J. Am. Chem. Soc.*, 41: 1866–1873 (1919).

amino acids protect malt amylase and pancreatic amylase from inactivation, and interprets his results from the viewpoint that these enzymes are protein compounds and accordingly the presence of amino acids in the digest should inhibit the destruction of the enzyme, if the destruction obeys the mass action equilibrium

$$\text{Protein} + \text{water} \rightleftharpoons \text{amino acids}$$

He accordingly interprets the protective action of amino acids, in the instances which he has studied, as the retardation of enzyme hydrolysis.

The evidence which he presents in his papers appears to substantiate his argument. On the other hand, it is difficult to believe that the inhibition of hydrolysis is the sole factor involved. Minute traces of ethylene gas dissolved in water protect [56] the fermentation enzymes of yeast against inactivation. The activity of zymase preparations was increased by bubbling ethylene through the solution, and solutions treated with ethylene maintained for a long period of time their ability to ferment glucose. The active fermenting system of zymase and sugar did not bring about the rapid inactivation of the enzyme which was present. Accordingly, Nord and Franke suggest that ethylene acts in this instance not as an activator but rather as a protector, and that in all probability the various experiments in the literature which have been reported as dealing with enzyme activation should be interpreted as experiments dealing with enzyme protection, the apparent increased activity being due to the inhibition of enzyme destruction. This view appears to be a logical one, assuming, of course, that the other physical factors in the environment have been kept constant. Undoubtedly, at least a part of the effect of hydrogen-ion concentration is due to an increase or decrease of the effective surface of the

Sherman, H. C., and Wayman, M., Effect of Certain Antiseptics upon the Activity of Amylases, *ibid.*, 43: 2454–2461 (1921). Sherman, H. C., and Walker, F., The Influence of Certain Amino Acids upon the Enzymic Hydrolysis of Starch, *ibid.*, 43: 2461–2469 (1921). Sherman, H. C., and Caldwell, M. L., A Study of the Influence of Arginine, Histidine, Tryptophane and Cystine upon the Hydrolysis of Starch by Purified Pancreatic Amylase, *ibid.*, 43: 2469–2476 (1921). Influence of Amino Acid in Protecting Amylase from Inactivation by Mercury, *ibid.*, 44: 2923–2926 (1922). Influence of Lysine upon the Hydrolysis of Starch by Purified Pancreatic Amylase, *ibid.*, 44: 2926–2930 (1922). Sherman, H. C., and Naylor, N. M., Influence of Some Organic Compounds upon the Hydrolysis of Starch by Salivary and Pancreatic Amylases, *ibid.*, 44: 2957–2966 (1922). Sherman, H. C., and Walker, F., Effect of Amino Acids in Retarding the Hydrolytic Decomposition of an Enzyme (Pancreatic Amylase), *ibid.*, 45: 1960–1964 (1923). Sherman, H. C., Caldwell, M. L., and Naylor, N. M., Influence of Tryptophane and Other Amino Acids upon the Stability and Enzymic Activity of Pancreatic Amylase, *ibid.*, 47: 1702–1709 (1925).

[56] Nord, F. F., and Franke, K. W., On the Mechanism of Enzyme Action. I. *Protoplasma*, 4: 547–595 (1928); II. *J. Biol. Chem.*, 79: 27–51 (1928).

colloidal enzyme, but at a fixed hydrogen-ion concentration and a fixed salt concentration it would be logical to interpret the effect of substances which activate enzymes in the same way that the action of protective colloids is interpreted.

This viewpoint appears to agree with that of Velluz,[57] who studied in particular the probable mechanism whereby small amounts of fatty acids are able to inactivate pepsin acting upon coagulated egg albumin. Table LXXXV shows certain of his results. In the third column

TABLE LXXXV

THE APPARENT RELATIONSHIP BETWEEN THE RETARDATION OF PEPTIC ACTIVITY DUE TO THE ADDITION OF SMALL AMOUNTS OF FATTY ACIDS AND THE SURFACE AREA COVERED BY A MOLECULE OF THE FATTY ACID (PEPSIN 0.25%, HCl 0.25%, COAGULATED EGG WHITE 0.5%, FATTY ACID 0.04%, TIME 16 HOURS) (FROM DATA OF VELLUZ)

Fatty Acid Added	Soluble Amino Nitrogen, mg. per 100 cc.	Area Covered by a Molecule of Fatty Acid (after Langmuir), $\times 10^{-16}$ sq. cm.
None	7.2	
Caproic	6.6	
Caprylic	6.3	
Capric	6.1	
Palmitic	5.8	21
Stearic	5.8	22
Oleic	2.8	46
Ricinolic	1.2	110
Linolenic	1.3	65

of the table are listed Langmuir's calculations of the surface areas covered by one molecule of the fatty acid. Velluz notes that, in general, there is a relationship between the effect of the fatty acid in inhibiting proteolytic activity and the area which is covered by a molecule of the fatty acid, and he notes that pepsin is apparently unable to attack the colloidal micelles of egg albumin when the micelles are partially or entirely covered by an oriented layer of fatty acid molecules. He tests this hypothesis by adding gum arabic and the fatty acid simultaneously to the pepsin-albumin system and finds that under these conditions there is little or no interference with proteolytic activity. He explains this by assuming that the fatty acid

[57] Velluz, M. L., Recherches sur l'action inhibitrice des acides gras et, en particulier des acides non saturés, sur les phénomènes diastasiques, *Bull. soc. chim. biol.*, 9: 483–500 (1927).

which is added becomes oriented upon the surface of the gum arabic, thus leaving the surface of the protein free to be attacked by the pepsin.

One other factor should be mentioned in the discussion of activation and inactivation, *i.e.*, the effect of the accumulated end products upon the course of the reaction. This has already been noted in the discussion of the kinetics of enzyme action. It seems logical to assume, at least until demonstrated to the contrary, that all enzyme reactions proceed to an equilibrium. Accordingly, as a reaction progresses and the concentration of the end products increases, one should expect according to the law of mass action that the reaction would be slowed up. We have already noted that we know far too little in regard to the nature of enzymes and of the variables which may be present in a given system to introduce necessary corrections to account for this retardation. The problem is complicated enough when we can demonstrate that only a single enzyme is involved. It may well be that our supposedly single-enzyme preparation contains in reality a mixture of enzymes. If these enzymes induce different reactions, it may be possible to demonstrate that our enzyme preparation is a mixture. However, it is not inconceivable that different enzymes may induce identical reactions or at least reactions which are essentially indistinguishable from each other by our present methods. That this is a probability is evidenced by the paper of Kuhn and Münch.[58] These authors studied twelve sucrase preparations derived from twelve different strains of yeast, and were able to divide these twelve preparations into four groups. The method employed was to study the influence of added glucose or fructose (the products of hydrolysis of sucrose) upon the rate of sucrose hydrolysis. The four sucrases which they identified were: (1) those sucrases whose rate of reaction was slowed up by the addition of β-glucose much more than by the addition of α-glucose; the addition of fructose likewise slowed up the rate of reaction of this group; (2) those sucrases whose rate of reaction was slowed up by the addition of fructose but whose rate of reaction was not altered by the addition of either α- or β-glucose; (3) those sucrases whose rate of reaction was retarded by the addition of either α- or β-glucose but was not altered by the addition of fructose; and (4) those sucrases whose rate of reaction was altered equally by the addition of either α-glucose, β-glucose, or fructose. Table LXXXVI shows examples of their data.

It is evident from these studies that the literature on "invertase" is filled with papers where one investigator has been discussing the behavior of one enzyme and another investigator the behavior of a different enzyme, and it is therefore not surprising to find that

[58] Kuhn, R., and Münch, H., Über Gluco-und Fructosaccharase, *Z. physiol. Chem.* 150: 220–242 (1925).

TABLE LXXXVI

THE RETARDATION OF THE ACTION OF VARIOUS SACCHARASE PREPARATIONS BY THE ADDITION OF α-GLUCOSE, β-GLUCOSE, OR FRUCTOSE (0.2 *N* SUCROSE, 0.2 *N* HEXOSE, 25° C., *p*H 5.0) (DATA OF KUHN AND MÜNCH)

Saccharase No.	Type	Sugar Added—Retardation		
		α-Glucose, per cent	β-Glucose, per cent	Fructose, per cent
A	I	0.0	23.0	15.5
K	I	0.0	9.2	25.5
B	II	0.0	0.0	10.0
M	II	0.5	0.5	30.0
P	III	9.0	11.5	0.0
F	III	5.9	8.8	0.0
E	IV	26.0	28.0	25.0
D	IV	18.0	18.5	16.5

various investigators have expressed divergent views on enzyme problems.

That other enzyme preparations possibly represent mixtures is evidenced by Willstätter's studies [59] on emulsin from various sources tested on a variety of β-glycosides as substrates. The emulsins from the various sources hydrolyzed the various glycosides at very unequal rates. The "optimum" hydrogen-ion concentration for maximum activity for different preparations was found to vary as widely as *p*H 4.0 and *p*H 6.0. Similarly Willstätter [60] differentiated six amylases as present in fresh leucocytes by studying activity behavior under variable experimental conditions with starch and glycogen as substrates. It appears therefore that many of the enzymes with which we are at present familiar may represent mixtures, and when the final story is told we may have an interclassification of enzymes.

Enzyme preparations are readily inactivated by traces of the heavy metals. This is notably true of such metals as copper, silver, mercury, and lead. We have noted that these same metals "poison" the hydrogen electrode, and we have similarly noted that at least three of these metals are the "cumulative poisons" in respect to living organisms, and further that they have a profound influence upon both lyophilic and lyophobic colloid systems. It seems logical, therefore, to interpret the effect of these metals on the inactivation of enzyme systems in

[59] Willstätter, R., and Csanyi, W., Zur Kenntnis des Emulsins, I, *Z. physiol. Chem.*, 117: 172–200 (1921); Willstätter, R., and Oppenheimer, G., II, *ibid.*, 121: 183–194 (1922).

[60] Willstätter, R., and Rohdewald, M., Über die Amylasen der Leukocyten, *Z. physiol. Chem.*, 203: 189–240 (1931).

terms of colloid chemistry and to postulate that a pronounced reduction of active surface is brought about by the presence of these metal ions, or that the ions are irreversibly adsorbed upon the active areas of the enzyme surface and thus block access of the substrate to these active areas. The latter hypothesis is substantiated by the observations of Quastel (*loc. cit.*) that the active centers on the bacterial mosaic were inactivated by mercury ions and that reactivation was possible when the inactive mosaic was treated with hydrogen sulfide. Apparently the mercury ion was irreversibly adsorbed but the mercury sulfide which was later formed was not specifically attracted to the active areas and moved out of the interface, thus regenerating the active areas.

Specific Enzyme Reactions.—We have already noted in this chapter that the literature dealing with specific enzymatic reactions is exceedingly voluminous, and that from time to time in the preceding chapters we have discussed certain interrelationships between enzymes and substrates. In view of these facts, it seems wise to close the discussion of enzymes at this point, having emphasized only some of the broader principles which are involved. To extend the discussion so as to make this text adequate in the field of enzymes would be to extend it beyond the purposes for which the volume is intended. For detailed discussion of the various enzyme systems the reader is referred to the extensive monographs of Oppenheimer or Euler, already noted. For a more brief but most excellent presentation the recent volume by Tauber [61] is strongly recommended.

[61] Tauber, Henry, Enzyme Chemistry, John Wiley & Sons, New York (1937).

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